Transforming growth factor –β signaling through Smad3 in allergy

Studies in the mechanisms of asthma, atopic dermatitis and allergic contact reactions

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ACADEMIC DISSERTATION

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Contents

1. LIST OF ORIGINAL PUBLICATIONS

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II Anthoni M, Wang G, Leino MS, Lauerma AI, Alenius HT, Wolff HJ. Smad3 -signalling and Th2 cytokines in normal mouse airways and in a mouse model of asthma. Int J Biol Sci 2007;3:477-85.

III Anthoni M, Fyhrquist-Vanni N, Wolff H, Alenius H, Lauerma A. Transforming growth factor-β/Smad3 signalling regulates inflammatory responses in a murine model of contact hypersensitivity. Br J Dermatol 2008; 159: 546-554.

IV Anthoni M, Fyhrquist N, Lehtimäki S, Alenius H, Wolff H, Lauerma A. Smad3 regulates dermal cytokine expression and specific antibody production in murine responses to a respiratory chemical sensitizer. (Submitted)

The publications are referred to in the text by their Roman numerals.

2. ABBREVIATIONS

ACD	allergic contact dermatitis
AD	atopic dermatitis
AHR	airway hyperresponsiveness
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BMP	bone morphogenetic protein
CCL	C-C family chemokine ligand
CCR	C-C family chemokine receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CHS	contact hypersensitivity
Co-Smad	common-partner Smad
cpm	counts per minute
CTL	cytotoxic T cell
CXCL	C-X-C family chemokine ligand
CXCR	C-X-C family chemokine receptor
DC	dendritic cell
DNA	deoxyribonucleic acid
DNFB	2,4-dinitrofluorobenzene
DTH	delayed type hypersensitivity
EC	epicutaneous
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial cell-derived neutrophil attractant -78
Foxp3	forkhead box P3
GCP-2	granulocyte chemotactic protein -2
HPF	high power field
HPLC	high-performance liquid chromatography
I-309	inducible-309
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	interferon inducible protein-10
I-Smad	inhibitory Smad
I-TAC	IFN-γ-inducible T-cell chemoattractant
JNK	Jun N-terminal kinase
kDa	kilo Dalton
KO	knock out
LAP	latency associated protein
LC	Langerhans cell
LIX	lipopolysaccharide induced CXC chemokine

LLNA	local lymph node assay
LTBP	latent TGF-β binding protein
MAD	mothers against decapentaplegic
MAPK	mitogen-activated protein kinase
MCh	methacholine
MCP	monocyte chemoattractant protein
MDC	monocyte-derived chemokine
MGG	May-Grünwald-Giemsa
MH	mad homology
MHC	major histocompatibility complex
MIG	monokine induced by gamma interferon
MIP-1α	macrophage inflammatory protein 1-α
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MSA	mouse serum albumin
OVA	ovalbumin
PAS	periodic acid Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Penh	enhanced pause
PI3K	phosphatidylinositol-3-kinase
PMN	polymorphonuclear neutrophil leukocyte
PP2A	protein phosphatase 2A
RANTES	regulated upon activation normally T-cell expressed and secreted
RNA	ribonucleic acid
RQ	relative quantity
R-Smad	receptor activated Smad
SDS	sodium dodecyl sulfate
SDF	stromal cell-derived factor
SMAD	The SMAD proteins are homologs of both the drosophila protein, mothers against decapentaplegic (MAD) and the *C. elegans* protein SMA. The name is a combination of the two.
SPF	specific pathogen free
TARC	thymus- and activation-regulated chemokine
TβR(I)	type I TGF-β receptor
TβR(II)	type II TGF-β receptor
TβR(III)	type III TGF-β receptor
TCR	T-cell receptor
TGF-β	transforming growth factor -β
Th	T helper
TMA	trimellitic anhydride
TNF	tumor necrosis factor
WHO	World Health Organization
WT	wild type

3. ABSTRACT

Transforming growth factor (TGF)-β is an important immunomodulatory and fibrogenic factor that regulates cellular processes in injured and inflamed skin. TGF-β signals are delivered from the cytoplasm to the nucleus by TGF-β signal transducers called Smads. Smad3 is a major signal transducer in TGF-β -signalling that controls the expression of target genes in the nucleus in a cell-type specific manner.

In this thesis, the role of TGF-β-Smad -signalling pathway using Smad3 -deficient mice in murine models of allergic diseases, atopic dermatitis, asthma and contact dermatitis was examined.

Dermatitis was induced in mice by epicutaneous application of ovalbumin (OVA) applied in a patch to tape-stripped skin (I). The thickness of dermis was significantly reduced in OVA-sensitized skin of Smad3-/- mice. The defect in the dermal thickness accompanied with the decrease in the expression of mRNA for proinflammatory cytokines IL-6 and IL-1β in the OVA-sensitized skin. In contrast, the number of mast cells was significantly increased in OVA sensitized skin of Smad3-/- mice and they also exhibited elevated levels of OVA-specific IgE.

The role of TGF-β/Smad3 -signalling in cytokine homeostasis was studied in the airways of naive mice and for the regulation of bronchial hyperreactivity and inflammation using a murine model of asthma. Compared to wild type mice, naive (unmanipulated) Smad3-/- mice exhibited significantly increased levels of proinflammatory and Th2 cytokines as well as the Th2 associated transcription factor GATA-3 in the lung tissue and bronchoalveolar lavage (BAL). In the asthma model, mucin secretion and airway hyperresponsiveness (AHR) after allergen exposure were significantly increased in the Smad3-/- mice as compared to WT mice. OVA-specific IgE, IgG_1 and IgG_{2a} levels were increased in Smad3-/- and WT mice. IL-4 levels were equally elevated but IL-13 levels were decreased in the airways of OVA sensitized Smad3-/- mice compared to the controls.

In the contact hypersensitivity studies (CHS), the skin and ears of Smad3 mice were treated with a contact allergen, oxazolone (III), or a respiratory allergen (IV), trimellitic acid anhydride (TMA). Topical exposure to oxazolone induced a significantly increased expression of CCL3/MIP-1α and CXCL5/LIX accompanied by increased neutrophil

infiltration in the skin of Smad3 deficient mice when compared to their wild type siblings. The mRNA expression for pro-inflammatory and Th2 cytokines were also significantly increased in Smad3-/- mice compared to wild type mice. Furthermore, topical exposure to oxazolone induced significantly increased expression of TGF-β and Foxp3 mRNA in Smad3-/- mice. However, Th1 cytokine IFN-γ, as well as Th1 type chemokines CXCL9/MIG and CXCL10/IP-10 were unaffected by the lack of Smad3, and the ear swelling responses to topically applied oxazolone were similar in Smad3 knockout mice as in wild type mice.

Topical exposure to TMA, as well as oxazolone, promoted proinflammatory and Th2 cytokine secretion in mice with a defect in TGF-β/Smad3 -signaling. In addition, the exposure to TMA upregulated mRNA expression for Th1 cytokines. The expression of IL-17 mRNA in the skin of TMA sensitized Smad3-/- mice was also increased pointing to an involvement of Smad3 in the progress of IL-17-induced tissue inflammation. TMA, like OXA, induced significantly increased expression of CCL3/MIP-1α and CXCL5/LIX in the skin of Smad3 deficient mice when compared to their wild type siblings.

In conclusion, Smad3-pathway regulates allergen induced skin inflammation and systemic IgE antibody production in a murine model AD. The defect in Smad3 -signalling decreased Th2 cytokine (IL-13 and IL-5) mRNA expression in the lung, modulated allergen induced specific IgG1 response, and affected mucus production in the lung in a murine model of asthma. TGF-β / Smad3 -signalling contributed to inflammatory hypersensitivity reactions and disease progression via modulation of chemokine and cytokine expression and inflammatory cell recruitment, cell proliferation and regulation of the specific antibody response in a murine model of CHS. TGF-β modulates inflammatory responses - at least partly through the Smad3 pathway - but also through other compensatory, non-Smad-dependent pathways. In the future the Smad3 signalling pathway might be a potential target in the therapy of allergic diseases.

4. INTRODUCTION

Allergic diseases, such as atopic dermatitis, asthma, and contact dermatitis are complex diseases influenced by both genetic and environmental factors. It is still unclear why allergy and subsequent allergic disease occur in some individuals but not in others. Although the reason for the increased prevalence of allergic diseases, especially in industrialized countries, is unknown, the improved hygiene and better infection control have been suggested to have had a potential influence on the development of allergy (Strachan 1989, von Mutius 2007).

Activated Th2 lymphocytes and the involvement of cytokines such as IL-4, IL-5 and IL-13 are responsible for the eosinophil activation and IgE production crucial in allergic inflammation (Spencer *et al.* 2008, Incorvaia *et al.* 2008). Reduced microbial exposures (or other environmental stimuli) in early life have been suggested to shift the polarization of allergen-specific T-cell memory towards the Th2 instead of the Th1 immune response (Chang and Pan, 2008).

However, the disturbance of the Th1/Th2 balance cannot explain on its own the dysregulated immune system involved in allergy; in addition some other mechanisms may be involved by T-regulatory cells. Many types of T-regulatory cells have been identified, most of these are recognized for their production of IL-10 and transforming growth factor-β (TGF-β). An immune suppression of the T-regulatory cells has been proposed to be involved in the Th2-skewed immune response among atopic individuals (Bach 2002, Romagnani 2004). It is known that allergy results from a breakdown of tolerance to allergens. Tolerance re-induction may be used as a tool since it can be considered as an immunotherapeutic in allergic diseases. An immunosuppressive cytokine, TGF-β is involved, in immunological tolerance and the regulation of the immune response to allergens (Akdis 2006, Taylor *et al.* 2006).

TGF-β signals are delivered from the cytoplasm to the nucleus by TGF-β signal transducers called Smads. Smads are intracellular proteins, transcription factors of 42-65 kDa in mass (Massague 1998, Moustakas *et al.* 2001). Smad3 is the crucial signal transducer in TGF-β -signalling (Wang *et al*. 2005) and it controls the expression of target genes in the nucleus in a cell type specific manner. Understanding the effects of the TGF-

β signalling pathway in immune system and in disease models can help in elucidating the multilevel effects of TGF-β. At present the role and the functions of Smad3 in allergy are largely unknown. In this thesis, the significance of the Smad3 signal transducer was investigated in the murine models of atopic dermatitis, asthma, and allergic contact reactions.

5. REVIEW OF THE LITERATURE

5.1 Allergy and allergens

Allergy is defined as disease following a response by the immune system to an otherwise innocuous antigen (Janeway *et al.* 2005). It is a hypersensitivity reaction, initiated by specific immunologic mechanisms (Johansson *et al.* 2004). The term *hypersensitivity* describes objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons (Johansson *et al.* 2004). Hypersensitivity can be antibody-mediated or cell-mediated.

Hypersensitivity reactions have been classified into four types by Coombs and Gell (Coombs and Gell, 1963): type I responses are mediated by IgE, which induces mast cell activation. Type II (directed against cell-surface or matrix antigens) and type III reactions (directed against soluble antigens) are mediated by IgG. IgG antibodies to environmental antigens are commonly found without causing any signs or symptoms (Johansson *et al.* 2004).

In the non-IgE-mediated allergy, also allergen-specific lymphocytes can mediate the inflammation, as is the case in allergic contact dermatitis. Type IV hypersensitivity reactions are T-cell mediated and can be subdivided into three groups depending on the type of the cell reacting (T helper1, T helper 2 or cytotoxic T cells)(Janeway *et al.* 2005).

Allergic inflammation is the inflammation produced in sensitized subjects after exposure to a specific allergen(s). A single allergen exposure produces an acute reaction, known as an early-phase reaction or a type I immediate hypersensitivity reaction, in many cases followed by a late-phase reaction. If the exposure to allergen is persistent or repetitive, chronic allergic inflammation develops, with associated tissue alterations (Galli *et al.* 2008). In predisposed individuals, initial exposure(s) of professional antigen-presenting cells to allergen causes the activation of allergen-specific Th2 cells and IgE synthesis, known as allergic sensitization. Subsequently exposures to allergen lead to the infiltration of inflammatory cells causing early allergic responses and late allergic responses. Early-phase reaction is an IgE-mediated and it can occur within minutes of allergen exposure. Reactions can be localized (for example acute asthma attacks, urticaria, gastrointestinal reactions in food allergies) or systemic (anaphylaxis). IgE bound to FcεRI on mast cells

and basophils is crosslinked by allergen resulting in the release of the cells' diverse preformed and newly synthesized mediators (histamine, cysteinyl leukotrienes and cytokines) leading to vasodilation, increased vascular permeability with oedema and acute changes in the function of affected organs (urticaria, vomiting, bronchoconstriction, airway mucus secretion). The released mediators also cause the infiltration and the activation of inflammatory cells which contribute to the development of late-phase reactions (Galli *et al.* 2008, Holgate and Polosa, 2008). Chemokines released by mast cells and other cell types direct the recruitment of inflammatory cells that contribute to the late allergic response characterized by accumulated Th2 cells and eosinophils. Cells release pro-inflammatory mediators including cysteinyl leukotrienes and basic proteins (cationic proteins, eosinophil peroxidase, major basic protein and eosinophil-derived neurotoxin, cytokines IL-3, IL-5, IL-13). Th1-cell responses might also be responsible in some pathogenic features in chronic forms of atopy, including epithelial apoptosis and smooth-muscle-cell activation (Holgate and Polosa, 2008). Late-phase reaction develops 2-6 h, peaks 6-9 h after allergen exposure and resolves in 1-2 days. Skin late-phase reactions involve oedema, pain, warmth and erythema (redness). In the lungs, these reactions are characterized by airway narrowing and mucus hypersecretion. The local recruitment and activation of Th2 cells, eosionophils, basophils and other leukocytes occurs as well as persistent mediator production by resident cells (such as mast cells). Mediators that initiate late-phase reactions are thought to be derived from T cells (either resident or recruited to the sites of allergen challenge) that recognize allergen-derived peptides or resident mast cells activated by IgE and allergen (Galli *et al.* 2008). Regulatory T cells may suppress Th2 responses by secreting TGF-β and IL-10. In chronic allergic inflammation large numbers of innate and adaptive immune cells (in the form of leukocytes) are present at the affected site. In addition, there are substantial changes in the extracellular matrix and alterations in the number, phenotype and function of structural cells in the affected tissues (Galli *et al.* 2008).

Allergens are antigens that elicit hypersensitivity or allergic reactions (Janeway *et al.* 2005). An antigen is any molecule that can bind specifically to an antibody or T-cell receptor. The name arises from the ability to generate antibodies. Allergens can enter the body via the skin, airways or gastrointestinal tract.

5.1.1 Proteins

Protein allergens originate from proteins with a variety of biological functions, including proteases, ligand-binding proteins, structural proteins, pathogenesis-related proteins, lipid transfer proteins, and calcium-binding proteins (Chapman *et al.* 2007, Russano *et al.* 2008). Biological function may directly influence the development of IgE responses and lead to allergy and may initiate inflammatory responses in the lung that are associated with asthma (Chapman *et al.* 2007, Shakib *et al.* 2008). According to the protein family database analysis, the universe of protein allergens comprises more than 120 distinct protein families (Chapman *et al.* 2007).

5.1.2 Chemicals

5.1.2.1 Contact chemical allergens

Contact allergens are usually of small molecular weight and lipophilic enabling them to penetrate the outer skin layers. Due to their small size, they must bind to larger proteins to become allergenic (haptens; from the Greek verb haptein, to fasten). Contact allergens can be different types of compounds: metals (nickel, cobalt, chromate), preservatives (formaldehyde, parabens, methyl(chloro)isothiazolinone), fragrances, medicaments (neomycin, benzocaine, ethylene diamine), adhesives (epoxy resin), rubber (mercaptobenzathiazole, N-isopropyl-N-phenylediamine), plants (Basketter et al, 1999). Contact allergens commonly have irritant properties. In order to achieve effective skin sensitisation, a chemical allergen is transported by cutaneous dendritic cells from the skin in sufficient amounts and in an immunogenic form to the draining lymph nodes. In the draining lymph nodes, responsive T lymphocytes become activated and are induced to divide and differentiate.

5.1.2.2 Respiratory chemical allergens

While many chemicals are known to induce allergic contact dermatitis, fewer chemicals like diisocyanates, acid anhydrides (such as trimellitic anhydride, TMA), platinum salts and reactive dyes are associated instead with sensitisation of the respiratory tract (Botham *et al.* 1989, Briatico-Vangosa *et al.* 1994).

Chemical respiratory allergens are associated with selective Th2-type immune responses, whereas contact allergens, which are considered not to cause sensitisation of the respiratory tract, stimulate immune responses of preferential Th1 cell activation in mice (Kimber and Dearman, 1997).

5.2 Molecular basis of allergic reactions

The immune system consists of the adaptive and innate elements of the immune response. Innate immunity is responsible for the immune responses in early phases of the infection. It is present in all individuals at all times and it does not increase with repeated exposure to a given pathogen. Adaptive immunity or adaptive immune response is the response of antigen-specific lymphocytes to an antigen, developing also an immunological memory. In contrast to innate immunity, adaptive immune responses are generated by clonal selection of antigen-specific lymphocytes (Janeway *et al.* 2005).

Innate immune responses appear to be less critical in determining the outcomes of allergic diseases compared with their protective role against pathogens. However, the development of the adaptive immune responses may be affected by the interaction of the innate response to allergens. The cells responding in innate immunity are often present in the likely sites of allergen exposure, including skin and respiratory tract (Leggat *et al.* 2008).

5.2.1 Lymphocytes and antigen presenting cells

Lymphocytes are crucial in the development of the immune response in allergy. Lymphocytes are divided to two major types, T and B lymphocytes.

B cells are generated in the bone marrow throughout life. The antigen receptor on B lymphocytes, the B cell receptor, is a cell-surface immunoglobulin. After activation by an antigen, B cells differentiate into cells producing antibody molecules of the same antigen specificity as this receptor (LeBien and Tedder, 2008).

T cells develop in the thymus. They are defined by heterodimeric receptors (α:β or γ:δ heterodimeric receptor) associated with the proteins of the CD3 complex. (Janeway *et al.* 2005). Adaptive immune responses are initiated by T cells bearing alpha-beta (α-β) T cell antigen receptors (TCRs), which make up the majority of T cells.

Two classes of α-β T lymphocytes, CD4 and CD8, are involved in adaptive immune responses. They are distinguished by the expression of the cell surface co-receptor, their interaction with different MHC molecules, and by their different functions. CD4+ T cells, T helper (Th) cells recognize antigen presented by class II MHC molecules, present only on antigen-presenting cells (APCs), such as dendritic cells, B cells, and macrophages. APCs take up the antigen and process it (proteins into peptides) in endocytic vesicles, which are presented on the cell surface bound to class II MHC molecules (Arnold and Mannie, 1999, Nolte-'t Hoen *et al.* 2004). CD8 cytotoxic T (Tc) cells recognize antigen presented by class I MHC molecules (Cohn and Ray, 2003).

In the peripheral lymphoid tissues (lymph nodes, spleen, mucosal lymphoid tissues), T cells encounter the foreign antigens bearing APCs. Dendritic cells are the primary APCs that activate naive T cells. The processing of antigen leads to the maturation of immature DCs to mature DCs, which then leave the mucosal surface and migrate to local lymphoid tissues. After contacting its specific antigen on an APC in the peripheral lymphoid tissue, the CD4+ T cell stops migrating back into the bloodstream. Two signals are required for the initiation of T cell activation: 1) TCR recognition of an MHC class II peptide and 2) a simultaneous co-stimulatory signal delivered by the same APC. Subsequently, the T cell enters the G1 phase of the cell cycle and starts producing interleukin-2 (IL-2), which is an essential T cell autocrine growth factor stimulating the T cell to go through the cell cycle and to perform clonal expansion (Cohn and Ray, 2003). The result is a population of effector cells with the identical TCR specificity to the parental cell. If a CD4+ T cell recognizes its specific antigen on an APC (signal 1) without a co-stimulatory signal, the cell becomes anergic or unresponsive because it is not able to proliferate due to the lack of IL-2 production (DeSilva *et al.* 1991).

5.2.2 Other inflammatory cells

Mast cells, basophils and eosinophils are essential cellular components of allergic inflammation.

The mast cell is a tissue-based inflammatory cell of bone marrow origin that responds to signals of innate and acquired immunity with immediate and delayed release of histamine

and other inflammatory mediators to secrete cytokines such as TNF-α thereby initiating a local inflammatory response. Mast cells are linked to the pathogenesis of allergic diseases via their ability to be activated through FcεRI-bound antigen-specific IgE. Mast cell activation occurs only when the bound IgE is cross-linked by multivalent antigen. This signal activates the mast cell to release in seconds the contents of its granules. Degranulation releases the stored histamine, causing a local increase in blood flow and vascular permeability leading quickly to an accumulation of fluid and blood proteins, including antibodies, in the surrounding tissue. Soon, during the next few minutes to a few hours, polymorphonuclear leukocytes infiltrate and, later, also macrophages, eosinophils, and effector lymphocytes (Janeway *et al.* 2005). Mast cells increase several-fold in association with IgE-dependent immediate hypersensitivity reactions, including urticaria and asthma, connective tissue disorders, and infectious diseases (Prussin and Metcalfe, 2006).

Mast cells produce pre-formed mediators, such as histamine and serine proteases, newly synthesized lipid mediators, such as prostaglandin (PG) D_2 and the leukotriene (LT) C_4. Skin mast cells produce more PGD_2 than LTC_4, whereas the opposite is produced by mast cells from the lung. PGD_2 and LTC_4 are bronchoconstrictors and PGD_2 is a neutrophil chemoattractant (Prussin and Metcalfe, 2006).

TNF-α, IL-4, IL-5, IL-6, IL-8 are the major cytokines produced by mast cells. Mast cells also produce chemokines, such as CCL3 (macrophage inflammatory protein 1α) (Prussin and Metcalfe, 2006). Participation of mast cell activation in the initiation of the immediate hypersensitivity reaction occurs in several forms, e.g. in the skin as erythema, edema, and itch; in the upper airways as sneezing, rhinorrhea, and mucous secretion; in the lungs as cough, bronchospasm, edema, and mucous secretion. There are also a late-phase reactions, i.e. the initial symptoms can be followed 6 to 24 hours later by leukocytic influx and a persistent edema. Mast cells might contribute to the downregulation of the allergic response producing and the release of IL-1 receptor antagonist, heparin, and other molecules with anti-inflammatory properties (Prussin and Metcalfe, 2006).

Basophils are granulocytes sharing common features with mast cells, such as FcεRI expression, Th2 cytokine expression, and histamine release. After activation, basophils

rapidly start to produce IL-4 and IL-13, indicating that they play a role in allergic inflammation (Prussin and Metcalfe, 2006).

Eosinophils are granulocytic leukocytes that are produced in bone marrow from pluripotential stem cells. Eosinophils are primarily found in tissues, especially in the connective tissue immediately underneath respiratory, gut, and urogenital epithelium (Janeway *et al.* 2005). It has been estimated that in humans the eosinophil tissue/blood ratio is about 100:1 (Kita *et al.* 2003). The specific localisation of eosinophils, as well as mast cells, in mucosal tissues refers to the role in mucosal immune response.

Tissue eosinophilia is often found at inflammatory sites and peripheral blood eosinophil counts become increased in allergic disease and asthma. Eosinophils are thought to play a central role in allergic disease and asthma, with eosinophil mediators, such as cytokines and major basic protein (MBP), evoking mucosal inflammation and consequent bronchial hyperresponsiveness in asthma (Adamko *et al.* 2003).

5.2.3 Immunoglobulin E

IgE is an immunoglobulin isotype composed of two types of protein chain: heavy chains and light chains. Antibodies of different immunoglobulin isotype are produced in an immune response in distinct places and have distinct effector functions. IgE antibody is present only at very low levels in blood or extracellular fluid but is bound avidly by receptors on mast cells that are found just beneath the skin and mucosa, and along blood vessels in connective tissue. Antigen binding to this cell-associated IgE triggers mast cells to release powerful chemical mediators that induce reactions, such as coughing and sneezing (Janeway *et al.* 2005). Two signals are required to trigger IgE synthesis. Signal 1 is provided by IL-4 or IL-13, which activate transcription at a specific immunoglobulin locus. The engagement of CD40 on B cells by the CD154 expressed on T cells provides the second signal required for switching on IgE. T cells are the principal source of these signals (Cameron and Vercelli, 2003).

Most patients with allergic symptoms from mucosal membranes in the airways and gastrointestinal tract, express IgE antibodies to allergens and the allergy is said to be IgE-mediated. Total serum IgE concentrations tend to be higher in allergic individuals

compared to nonallergic adults and children, but the diagnostic relevance is limited as a screening test for allergic disease (Klink *et al.* 1990, King *et al.* 2004). Allergen-specific IgE antibodies in atopic individuals originate from a broad variety of B cells reflecting the activation of multiple B-cell clones during allergen sensitization (Eibensteiner *et al.* 2000).

5.2.4 Cytokines and chemokines

Cytokines are proteins made by cells that influence the behavior of other cells. They function via specific cytokine receptors on the cells that they affect (Janeway *et al.* 2005). Cytokines participate in the regulation of immune responses by regulating critical cell functions, i.e. growth, differentiation and activation. Cytokines are involved in antigen presentation, bone marrow differentiation, cellular recruitment and activation, adhesion molecule expression, and acute-phase responses (Borish and Rosenwasser, 2003).

Chemokines are small chemoattractant proteins that stimulate the migration and activation of cells, especially phagocytic cells and lymphocytes (Janeway *et al.* 2005). The family of chemokines consists of around 50 small proteins. Four chemokine groups have been classified on the basis of the arrangement of their amino-terminal cysteine residues. Most of the chemokines belong to the CC or CXC classes, where the two N-terminal cysteines are adjacent or have a single amino acid separating them, respectively (Zlotnik and Yoshie, 2000). A C class has a single amino-terminal cysteine and in the chemokines of a CX3C class the two cysteines are separated by three residues (Bazan *et al.* 1997).

Table 1 *Common cytokines and their effects in allergic diseases.*

Cytokine	**Examples of sources**	**Stimuli for production**	**Effects**
IL-1β	monocyte/macrophage, neutrophil	endotoxin,bacteria, viruses, TNF-α	Adhesion molecule and TNF-α production, proliferation, chemokine production, activation
IL-6	monocyte/macrophage, lymphocyte, mast cell, eosionophil, epithelial cell, fibroblast	endotoxin, IL-1, TNF, histamine	Stimulates acute phase protein synthesis in liver
TNF-α	monocyte/macrophage, many cells	endotoxin, bacteria, viruses, IL-1	Enhanced proliferation, adhesion molecule expression, (upregulates endothelial and epithelial adhesion molecules)
IFN-γ	Th1 lymphocyte	IL-2 and some other growth factors	Promotes class switching to IgG2a; inhibits switching to IgE, inhibits action of IL-4
IL-4	T cell, mast cell	antigen activation of T cell receptor, cross-linkage of FcεRI	Growth and activation, production of IL-6, TNF, switch factor for IgE and IgG1 (Th2 cell differentiation and IgE synthesis)
IL-5	lymphocyte, eosinophil, mast cell	antigen activation of T cell receptor	Proliferation and activation (eosinophil development and survival)

IL-13	Th2 lymphocyte, mast cell, eosinophil	T cell receptor	Mucus production, eotaxin production, induction of matrix proteases, many effects similar to IL-4
IL-10	lymphocyte, monocyte/macrophage, mast cell	T cell receptor, endotoxin for monocytes and mast cells	Inhibitory actions on antigen presentation, promotes tolerance
TGF-β	T cells, macrophages, B cells, fibroblasts and mast cells	Autoinduction after injury IL-1, vitamin D3	Immunosuppressive effects. Inhibits the production of TNF-α, and responses to IL-12, activation of macrophages, migration of cells. Promotes wound healing and excessive fibrosis. Regulates the adhesiveness of cells; increased synthesis of extracellular matrix protein expression, regulation of cell growth, differentiation and apoptosis.
IL-17	T cells (IL-17A, IL-17F) Expressed in peripheral and immune tissues (IL-17B) Nervous system and skeletal muscle (IL-17D) Peripheral tissues (IL-17E)	Upregulated in inflammatory conditions. IL-23, TGF-β, IL-6 induce the production. Thymus specific nuclear receptor, ROR-γ, directs differentiation of IL-17-producing T cells.	Induces and mediates proinflammatory responses. Induces the production of cytokines (IL-6, IL-1β, TNF-α, TGF-β), chemokines (IL-8, MCP-1),prostaglandins from many cell types (fibroblasts, endothelial cells, epithelial cells, keratinocytes, macrophages, referred to airway remodeling.

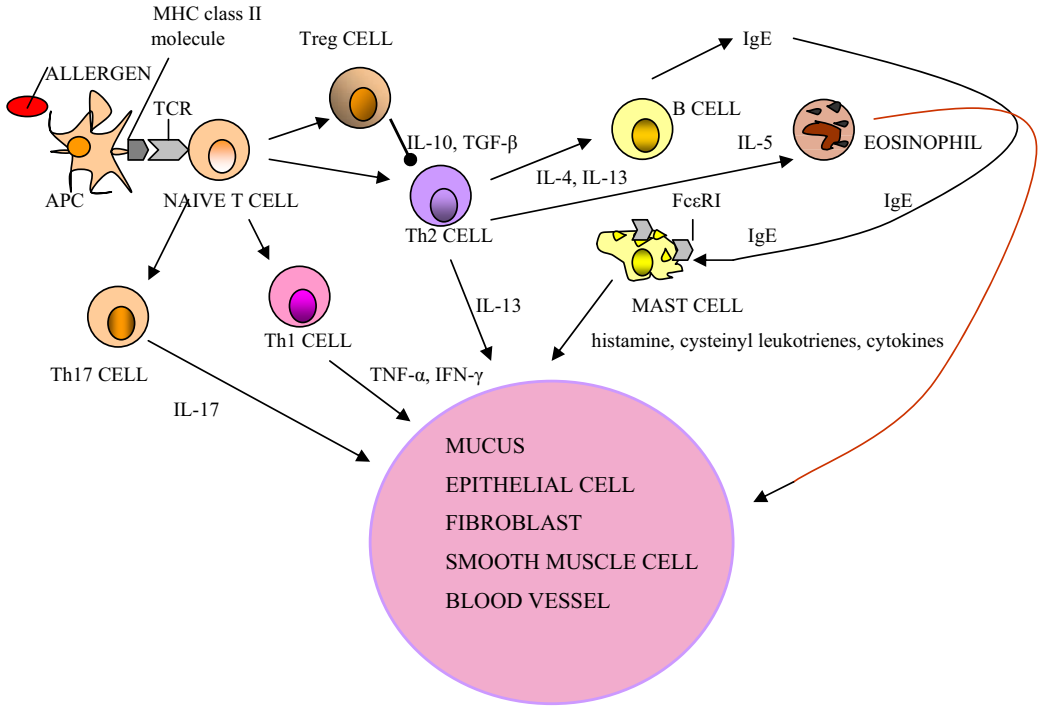

Figure 1 ***Mechanisms of allergic inflammation reaction.*** *The activation of allergen-specific Th2 cells and IgE synthesis by exposure of professional antigen-presenting cells (APCs) to allergen in predisposed individuals. Subsequent exposures to allergen cause inflammatory cell recruitment and activation and mediator release. Within minutes of contact with allergen, IgE-sensitized mast cells degranulate releasing mediators (histamine, cysteinyl leukotrienes, cytokines) in sensitized individuals. Chemokines released by mast cells and other cell types direct the recruitment of inflammatory cells contributing to the late allergic response which is characterized by influx of eosinophils and Th2 cells. These cells secrete more inflammatory mediators, cytokines and chemokines.Regulatory T (Treg) cells are able to suppress Th2-cell responses by inhibitory cytokines IL-10 and TGF-β. Th17 cells secrete IL-17, which participates in the neutrophilic inflammatory events during disease exacerbation and in tissue remodelling (modified from Holgate and Polosa, 2008).*

Leukocytes possess cell-surface sensors for chemokines. Chemokines signal through G protein-coupled receptors and the repertoire of 19 receptors are differentially expressed on leukocytes depending on the type of leukocyte and its maturation stage. Several different chemokine receptors may be expressed at any one time, and these receptors can be broadly divided into two groups: those constitutively expressed by leukocytes and those which under inflammatory conditions are induced via receptor activation (Pease and Williams, 2006). Selective blocking of chemokine receptor by small-molecule antagonists represents a putative target with therapeutic significance since it may be possible to suppress the recruitment of particular leukocyte types in an allergic reaction.

5.3 Atopy, allergic skin diseases and asthma

5.3.1 Atopy

Atopy is a personal and/or familial tendency, appearing usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins. As a consequence, these atopic individuals can develop the characteristic symptoms of asthma, rhinoconjunctivitis, or eczema (Johansson *et al.* 2004). Atopy is a clinical definition of an IgE-antibody high-responder; it describes the genetic predisposition to become IgE-sensitized to allergens commonly occurring in the environment and to which everyone is exposed but to which a prolonged IgE-antibody response is not produced in the majority of the population (Johansson *et al.* 2004). Atopic diseases have recently increased to the extent that they can be viewed as an epidemic (Holgate 1999, Umetsu *et al.* 2002). For example, the incidences of three common syndromes of atopic origin (Johansson *et al.* 2001), asthma, atopic dermatitis and hayfever (allergic rhinitis), have almost doubled in industrialized countries over the past two decades (Umetsu *et al.* 2002, Cookson 1999). As many as 40 % of people in Western populations are now atopic (Janeway *et al.* 2005).

Table 2 *The cellular distribution of chemokine receptors and their principal ligands in allergic disease (modified from Pease and Williams, 2006).*

Cell type expressing chemokine receptor	**Receptors**	**Ligands**
T lymphocyte	CCR1	CCL3/MIP-1α, CCL5/RANTES, CCL7/MCP-3
	CCR3	CCL5/RANTES, CCL11/eotaxin, CCL13/MCP-4, CCL24/eotaxin-2,
	CCR4	CCL26/eotaxin-3
	CCR8	CCL17/TARC, CCL22/MDC
	CXCR3	CCL1/I-309 CXCL9/Mig, CXCL10/IP-10,
	CXCR4	CXCL11/I-TAC CXCL12/SDF-1α
B lymphocyte	CXCR3, CXCR4	
Eosinophil	CCR1, CCR3, CXCR4	
Monocyte	CCR1, CCR8, CXCR4	
Basophil	CCR1, CCR3, CCR4, CXCR4	
Mast cell	CCR1, CCR3, CCR4, CXCR3, CXCR4	
Dendritic cell	CCR1, CCR4, CXCR4	

I-309, inducible-309; IP-10, IFN-γ-inducible protein-10; I-TAC, IFN-γ-inducible T-cell chemoattractant; MCP, monocyte chemoattractant protein; MDC, monocyte-derived chemokine; Mig, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T cell expressed and secreted; SDF, stromal cell-derived factor; TARC, thymus- and activation-regulated chemokine.

5.3.2 Atopic dermatitis

Atopic dermatitis (AD) is a common chronic inflammatory skin disease characterized by dry, inflamed and itchy skin (Hanifin and Rajka, 1980). Various susceptibility genes, host environments, infectious agents, defects in skin barrier function, and immunologic responses interact in the pathophysiology of AD (Novak *et al.* 2003). One typical characteristic of AD skin inflammatory responses is the activation of T lymphocytes, dendritic cells, macrophages, keratinocytes, mast cells, and eosinophils (Akdis *et al.* 2006). AD usually starts in early childhood and its prevalence has been increasing during recent years in parallel with other atopic conditions, i.e. asthma, rhinitis and allergies. It is believed that both genetic and environmental factors play an important role in developing of atopic dermatitis (Spergel and Paller, 2003; Foroughi *et al*. 2005; Linneberg *et al*. 2006).

Most patients with AD have elevated levels of serum IgE and specific IgE antibodies to environmental allergens are evidence of the important role played by allergens in AD. The initial event in the development of allergic disease is the generation of allergen-specific CD4+ Th cells (Romagnani, 2004). In the acute phase of AD, skin lesions show a marked infiltration with the presence of activated CD4+ T cells within the epithelium. Th2 cells, once generated, characterize the inflammatory response expressing mRNA for Th2-type cytokines, IL-4, IL-5 and IL-13. Th2-associated cytokines are thought to induce allergen-specific IgE production by B cells, thereby promoting the sensitization to allergen. Th2 cytokines also induce the development and recruitment of eosinophils, production of mucus, and the contraction of smooth muscle (Romagnani, 2004).

However, in the chronic phase of the disease, Th1-type cytokines become highly expressed, with the IL-12 and IFN-γ mRNA -expressing cells predominating over Th2 cells (Fiset *et al.* 2006, Grewe *et al.* 1998, Grewe *et al.* 1998). Th1 cells might also effectively contribute to the effector phase in allergic diseases (Trautmann *et al.* 2000, Trautmann *et al.* 2002) or, alternatively, dampen allergic inflammation, depending on the stage of the inflammation or on the disease model (Finotto *et al.* 2002). The maintenance of chronic AD also involves the production of the Th1-like cytokines, as well as remodelling-associated cytokines, such as TGF-β1 and IL-11, expressed preferentially in

the chronic state of the disease (Toda *et al.* 2003). The severity of AD has been connected to the amount of thymus and activation-regulated cytokine levels (Hijnen *et al.* 2004). Supporting the migration of Th1 cells toward the epidermis, an intense upregulation of several chemokines, such as IFN-γ-inducible protein 10, monokine induced by IFN-γ, and IFN-γ-inducible α chemoattractant (Klunker *et al.* 2003), and fractalkine (Echigo *et al.* 2004), in keratinocytes has been demonstrated. The levels of macrophage-derived chemokine and thymus and activation-regulated cytokine are elevated in AD patients (Leung *et al.* 2004). These proteins also mediate the selective recruitment of Th2-cells expressing CCR4. In addition, upregulation of monocyte chemoattractant protein 4, eotaxin, and RANTES, participate in promoting the infiltration of macrophages, eosinophils, and T cells into acute and chronic AD skin lesions (Taha *et al.* 2000).

In addition to Th1 and Th2 cells, a subtype of T cells, regulatory-suppressor T cells (Tregs) with immunosuppressive functions and a cytokine profile distinct from either Th1 or Th2 cells have been identified. Treg cells have been shown to inhibit the development of allergic Th2 responses (Cottrez *et al.* 2000).

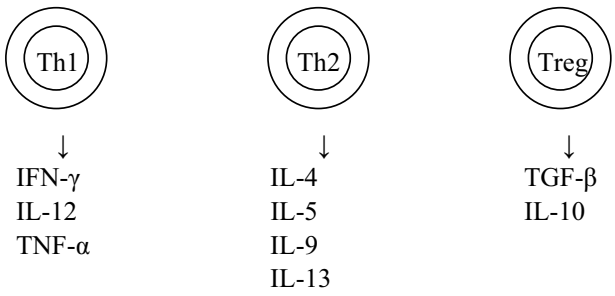

Figure 2 *Typical cytokines produced by Th1, Th2 and Treg cells. By direct secretion of cytokines, cells modulate the immune reaction at the site of inflammation.*

5.3.3 Contact hypersensitivity

Allergic reactions consist usually of two phases: the immediate reaction and delayed-type reaction. The hypersensitivity reactions that occur within minutes of exposure to antigen, are called immediate hypersensitivity reactions and are antibody mediated. The delayed-type hypersensitivity is a form of cell-mediated immunity elicited by the antigen in the skin and this is mediated by CD4 Th1 cells. The reaction appears hours to days after antigen is injected. Thus, a contact hypersensitivity reaction is a form of delayed-type hypersensitivity in which T cells respond to antigens that are introduced by contact with the skin (Janeway *et al.* 2005). In this thesis, the focus was on the cell mediated, chemical induced delayed-type hypersensitivity reaction, allergic contact dermatitis.

5.3.3.1. Allergic contact dermatitis

Contact dermatitis can be classified into the type IV hypersensitivity reaction, delayed-type hypersensitivity, reaction according to the classification by Coombs and Gell (Coombs and Gell, 1963). Tissue damage is evoked by Th1 cells activating macrophages or directly by cytotoxic T cell (CTL). A general view is that CHS is mediated by IFN-γ producing CD8+ T cells, while IL-2, IL-4 and IL-10 producing CD4+ T cells regulate the response.

Induction phase, sensitization, antigen recognition (afferent phase)

A hapten is a low-molecular-weight, chemically reactive, lipid-soluble molecule that crosses the stratum corneum. In the skin, it covalently binds to carrier proteins; cell surface or structural proteins on various cells (e.g. Langerhans cells (LC), keratinocytes) to form a hapten-carrier complex, the antigen. The antigen is processed by dendritic cells, known as Langerhans cells, at the site of epidermal penetration. The antigens are first digested into small peptides that bind to class II MHC molecules. Then, the peptide antigen/MHC complex is transported to the LC surface membrane. The antigen-bearing

LCs migrate from the skin, through the efferent lymphatics into the regional draining lymph nodes. LCs present the antigen to naive CD4+ helper T lymphocytes. Specific T cells subsequently proliferate clonally in the paracortical region of the lymph nodes. Finally, effector and memory T cells are released into the circulation (Mydlarski *et al.* 2003).

Elicitation phase, challenge (efferent phase)

During the elicitation phase, a sensitized individual is re-exposed to a hapten and the hapten-carrier complex is formed again. However, in this situation, the antigen is presented by LCs in the epidermis, dermis, and regional draining lymph nodes. Clonal proliferation of specific T lymphocytes follows the recognition of the peptide antigen/MHC complex by effector and memory T cells. Resident and activated cells release a barrage of cytokines and chemokines leading to inflammatory reaction within 48 hours after the re-exposure to the contact allergen (Cavani *et al.* 2000, Cavani *et al.* 2003, Mydlarski *et al.* 2003).

Irritants such as sodium dodecyl sulfate (SDS) cause keratinocyte damage and mononuclear cell infiltration. However, in contrast to haptens, they do not activate the immune cascade via the antigen presentation pathway.

5.3.4 Asthma

Allergic asthma results from immunologic reactions. IgE antibodies participate in the initiation of most allergic asthma cases (Johansson *et al.* 2004). Asthma can be classified into type I or type IV hypersensitivity reactions according to the classification by Coombs and Gell (Coombs and Gell, 1963). The type I type is characterized by IgE-mediated inducing mast cell activation.

Allergic asthma is a complex multifactorial disorder characterized by variable and reversible degrees of airway bronchoconstriction, hyperresponsiveness, mucus production, inflammation, and remodelling of the airways. Remodelling, as a feature of asthma, alters the size, mass or number of tissue components which occur in and around the trachea,

bronchi and bronchioles in the airways in response to injury and/or inflammation (Folli *et al.* 2008). CD4+ Th2 -type cells, which secrete IL-4, IL-5, and IL-13 are thought to contribute to the pathophysiological features of asthma. The inflammatory reaction is characterized by the influx of eosinophils, macrophages, neutrophils, CD4+ Th, and T cytotoxic (Tc) lymphocytes, mainly of type 2 cytokine phenotype (Th2 and Tc2) into airway epithelium and bronchial fluids (Henderson and Lodewick, 2003, Holgate, 2008). Cytokines, chemokines, adhesion molecules mediate airway inflammation and remodeling (Passalacqua and Ciprandi, 2008).

5.4. Experimental models in allergy research

Asthma

Sensitization to various allergens and following airway challenge with the specific allergen can produce the characteristic immunological, physiological, and pathological features of asthma in several species, including the mouse, rat, rabbit, guinea pig, dog, and nonhuman primates (Henderson and Lodewick, 2003). The timing, challenge, location, and type of allergen are important factors to be considered in selecting the experimental animal model. Several protocols describing the administration of ovalbumin (OVA) and other allergens (e.g. dust mite and fungal proteins) to induce allergen-specific pulmonary disease in animals have been described. Mouse develops IgE-mediated allergic responses and methacholine-induced changes in lung physiology, which mimic several aspects of human airway disease (Henderson and Lodewick, 2003).

Asthma models in mouse are typically short-term or long-term models. In the long-term model of chronic allergic airway inflammation, there is the development of fibrosis and airway remodeling. In this thesis, a short-term model was used.

In short-term models, mice are sensitized by intraperitoneal injection of an allergen (e.g. OVA) in alum adjuvant and mice are challenged with the specific allergen administered by aerosol, intratracheally, or intranasally. The repeated airway challenges with specific allergen are continued usually several weeks. Nonsensitized control mice are treated with alum alone via intraperitoneal injection, and only saline by airway administration. Airway administration of OVA promotes a Th2 phenotype in lymphocytes and these cells infiltrate the lungs of OVA-treated mice so that increased levels of total and OVA-specific

IgE can be detected. OVA-sensitized/challenged mice display mucus hypersecretion, eosinophilia and other inflammatory mediators in bronchoalveolar lavage (BAL) (Henderson and Lodewick, 2003). Allergen sensitization results in AHR, which can be measured by *in vivo* whole-body plethysmography (non-invasive technique). The degree of bronchoconstriction is calculated as enhanced pause (Penh), a dimensionless value, a junction of the proportion of the pressure signal from inspiration and expiration and of the timing of expiration (Hamelmann *et al.* 1997). The genetic background of the mouse is an important factor, since significant differences are observed in the ventilatory response between different mouse strains (Tankersley *et al.* 1994).

AD

One of the earliest animal models of AD was the NC/Nga mouse. The animals have been reported to develop *spontaneously* AD-like eczematous skin lesions when kept in an air-uncontrolled conventional room but not when maintained under specific pathogen-free (SPF) conditions (Matsuda *et al.* 1997). The clinical symptoms - itching, erythema, scaling dryness begin at the age of eight weeks.

In murine model of *protein* induced atopic dermatitis, repeated epicutaneous sensitization with ovalbumin (OVA) has been shown to cause thickening of epidermis and dermis, infiltration of T cells and eosinophils and elevated levels of IL-4, IL-5, IFN-γ mRNA, and airway hyperresponsiveness. The model can be developed in both BALB/c and C57BL/6 mouse strains (Spergel *et al.* 1998).

Transgenic and knockout mice form the third group of murine models of AD. IL-18-transgenic mice have been shown to develop AD-like skin lesions at about six months after birth under SPF conditions (Konishi *et al.* 2002).

Contact hypersensitivity

Murine models of dermal sensitization have been described (Dearman and Kimber, 1999, Regal *et al.* 2001). The significance of the mouse ear swelling test, first described by Gad *et al*, is to identify potential contact allergens on the basis of challenge-induced increases in the ear thickness in a sensitized animal (Gad *et al* 1986). A different strategy is used in the local lymph node assay (LLNA), a murine model, which is capable of detecting low molecular weight chemicals that cause contact hypersensitivity (ICCVAM, 1999, Sailstad

et al. 2001). In the standard LLNA assay, mice are treated topically on the back of both ears with a test substance for three days. Following two days of rest, the initiation of the hypersensitivity response is tested by injecting ^{3}H-thymidine into a tail vein, and then measuring the levels of radioisotope incorporated into the DNA of the cells of the lymph nodes draining the ears (Piccotti *et al.* 2006). Most, but not all, low molecular weight chemical allergens (both contact and respiratory) induce lymph node activation (Dearman *et al.* 1992). Those chemicals able to provoke a three-fold or greater increase in the proliferation of the draining lymph node cells compared with concurrent vehicle controls are classified as skin sensitizers (Piccotti *et al.* 2006). Chemical allergens have been differentiated from irritants by measuring the relative percentages of T and B cells in draining lymph nodes by flow cytometry (Gerberick *et al.* 2002).

It has been difficult to distinguish chemicals that cause respiratory hypersensitivity from the larger group of contact sensitizers. Contact hypersensitivity has been associated with the elaboration of Th1 cytokines without any increase in IgE, whereas respiratory hypersensitivity responses evoke increased Th2 cytokines and total serum IgE. This seems to be true with at least some chemicals, e.g. trimellitic anhydride (TMA) and 2,4-dinitrofluorobenzene (DNFB) (Dearman and Kimber, 1991,1992).

5.5. Transforming growth factor -β in allergic diseases

Transforming growth factor -β (TGF-β) is a multifunctional protein with widely diverse activities. Although much is known about its structure, expression, function, and receptors, the mechanisms of action and its role in allergy is still largely unknown.

5.5.1. Transforming growth factor -β

TGF-β first came to attention in 1981 as a 25 kD factor which was capable of inducing the growth of rodent fibroblasts in the presence of a cofactor, epidermal growth factor (EGF) (Moses *et al.* 1981, Roberts *et al.* 1981). TGF-β1 belongs to a transforming growth factor-beta superfamily, which consists of multifunctional proteins that can act both as cytokines and morphogens. TGF-β1 is the prototype of a subfamily containing 3 highly conserved isoforms (TGF-β1, TGF-β2, and TGF-β3). These three isoforms are secreted as latent

precursor molecules, which are usually complexed with latent TGF-β binding proteins, requiring activation into a mature form for receptor binding and subsequent activation of signal transduction pathways (Verrecchia and Mauviel, 2007, Keski-Oja *et al.* 2004). Each isoform has differential biological effects, with distinct tissue and developmental expression patterns. TGF-β1 is highly conserved between species. TGF-β1 is an 112 amino acid (12.5 kDa) protein, but physiologically, the ligand exists as a 25 kDa disulfide-linked homodimer. TGF-β1 has many roles and influences cellular function in many ways: cell proliferation, growth inhibition, differentiation, apoptosis, immunosuppression, angiogenesis, wound healing, extracellular matrix deposition, motility, and adhesion (Ruscetti *et al.* 1998). TGF-β1 stimulates the growth of mesenchymal cells, while inhibiting the growth of epithelial cells and many cells of the immune system. The physiological effects of TGF-β1 are cell-context specific; depending on the type, state of differentiation, and growth conditions of the cell. TGF-β1 interacts with and exerts its effects through two high-affinity cell surface receptors, the type III TGF-β receptor (TβRIII) and the type II TGF-β receptor (TβRII). TβRII ultimately complexes with the type I TGF-β receptor (TβRI), which activates the intracellular smad transcription factors. TGF-β binds to the receptor-complex on the cell surface, a complex of two pairs of subunits known as receptor type I (TβR-I, also known as ALK-5) and type II (TβR-II). Type III receptor or betaglycan, a membrane-anchored proteoglycan, aids this process by capturing TGF-β for presentation to the signalling receptors I and II (Massagué and Gomis, 2006). Furthermore, TGF-β binds to the endothelial-specific TβR-III-like receptor, endoglin, and to the type I TGF-β superfamily member ALK-1. In addition, TGF-β binds another small proteoglycan, decorin, an alpha-amyloid precursor protein and the serum glycoproteins fetoprotein and fetuin (Binkert *et al.* 1999).

Virtually every cell displays at least one of the TGF-β binding receptors (TβRIII and/or TβRII) and responds in some manner to TGF-β, which means that it is crucial that there is a strict regulation at the protein level (Massague *et al.* 1992). Uniquely among cytokines, TGF-β is predominantly secreted from cells as an inactive or latent complex that is unable to bind to its receptor (Pircher *et al.* 1986). TGF-β is synthesized as a 390 amino acid precursor and secreted as a latent complex that exists in one of two major forms: the small and large latent complexes. The small latent complex (~100 kDa) consists of the mature

TGF-β1 peptide at the carboxy-terminus covalently linked to latency-associated protein (LAP) at the amino-terminus. The large latent complex (~235-260 kDa) consists of the small latent complex and a latent TGF-β binding protein (LTBP), which is linked to the complex through a disulfide bond with the LAP (Miyazono *et al.* 1993). The LTBP appears to be important in the efficient secretion of TGF-β. The exact mechanism by which the latent form is converted to the active form remains unknown. It is thought that activation occurs through proteolytic cleavage or alterations that lead to changes in the conformation of the latent complex. TGF-β1 may also be further modified through phosphorylation and/or glycosylation. Activation of TGF-β1 is also cell-context specific (Lawrence 2001). The TGF-β1 protein is highly conserved in mammals: it is identical in man, pig, and cow, and differs by only one amino acid in mice. In general, the structure, activation, and signaling of murine TGF-β receptors are similar to those encountered in human receptors (Ruscetti *et al.* 1998).

Many factors are able to increase TGF-β1 transcription. In particular, TGF-β1 itself can induce TGF-β1 production in a positive-feedback loop. Importantly, mRNA levels of TGF-β1 are not an accurate indication of the protein production or protein activity, since the amount of active TGF-β1 is highly regulated and TGF-β1 can be activated without increasing protein synthesis. In addition, protein production can be increased, in the absence of transcription, through the binding of a regulatory protein to the 5'-untranslated region stem loop of TGF-β1 mRNA (Van der Velden and Thomas, 1999). Also, the activation of latent TGF-β1 can enhance TGF-β-mediated effects and there are estimates suggesting that only 2% to 5% of the latent protein is activated (Flaumenhaft *et al.* 1993). Thus, the activation of TGF-β1 is highly restricted and localized compared to the broad distribution of the latent complex. The availability of active TGF-β is likely transient and probably restricted to the local environment (Ruscetti *et al.* 1998). Varying levels of TGF-β1 expression have been linked in many diseases. TGF-β1 is often up-regulated in a number of different types of cancer. At the subcellular level, TGF-β1 can be found in the extracellular matrix, on the cell surface, and within endocytic vesicles (Letterio and Roberts, 1998, Makinde *et al.* 2007, Penheiter *et al.* 2002).

The TGF-β1 knockout mouse survives to birth, unlike the knockouts of the other two TGF-β isoforms. However, the TGF-β1 knockout animals die within 3 weeks with inflammatory cell infiltration in multiple organs and massive inflammation (Shull *et al.* 1992). TGF-β1 has also been shown to activate the p38, mitogen-activated protein kinases (MAPK), Jun N-terminal kinase (JNK), and ERK pathways, and Rho family members, although the exact mechanism is unknown (Nickl-Jockschat *et al.* 2007, Zhang *et al.* 2006, Derynck and Zhang, 2003).

5.5.2. Transforming growth factor -β in asthma and allergy

TGF-β has a significant role in the regulation of the allergen-induced immune response. It participates in the development and regulation of allergic and asthmatic inflammation. It appears to attenuate the inflammatory response and to contribute to the airway remodeling associated development of fibrosis.

TGF-β family proteins have multiple functions which in some cases can be conflicting - either stimulating or inhibiting - in affecting immune cell functions and in this way it has complex effects on the function and activity of immune cells (Meadows *et al.* 2006, Ito *et al.* 2006, Smeltz *et al.* 2005). Most, if not all, mature cells produce TGF-β, enhanced TGF-β expression occurs during tissue repair, bone remodelling, and inflammation (Wahl, 1992).
Also blood platelets store high levels of TGF-β delivering it among inflammatory cells at the sites of tissue injury (Wahl, 1994). TGF-β activates cellular mitogen-activated protein kinase signaling pathways, which crosstalk with Smad signaling and regulate growth, survival and motility of cells (Leivonen and Kähäri, 2007). TGF-β activates elastin transcription, which is important for lung development and postnatal maturation as well as for repair following injury (Kuang *et al.* 2007). TGF-β/Smad signaling mediates also the induction of ECM glycoproteins required for normal elastogenesis during lung injury and repair (Kuang *et al.* 2006).

Increased activation of the TGF-β-pathway has been observed in the skin and lung, especially during tissue injury and inflammation (Munger *et al.* 1999). Children with

atopic dermatitis exhibit a significantly higher proportion of a low TGF-β1 cytokine producing allele compared to healthy controls (Arkwright *et al.* 2001).

As stated above, the reaction of epidermal LCs to haptens or metals is the initial step in allergic contact sensitivity. It has been proposed that one way to study the biological role of epidermal LC in allergic contact sensitivity, is to substitute the immature epidermal LCs with TGF-β -treated dendritic cells; this has been claimed to provide a good *in vitro* model (Aiba *et al.* 2000). TGF-β contributes to the initiation step in the allergic contact sensitivity reaction.
It has been reported that TGF-β down-regulates TARC/CCL17 synthesis and in this way it can decrease the TARC-related inflammatory processes such as AD (Zheng *et al.* 2002).

TGF-β may decrease allergic inflammation by inhibiting IgE synthesis in IL-4-treated human B cells and by inhibiting the proliferation of mast cells. TGF-β has been shown to inhibit Th2 lymphocyte differentiation (Gorelik *et al.* 2000, Jutel *et al.* 2003). AHR and airway eosinophilia in murine asthma were reversed by T lymphocytes manipulated to overproduce TGF-β (Hansen *et al.* 2000).

The earliest studies linking TGF-β to inflammation modulation showed enhance TGFβ gene transcription and production in bronchoalveolar mononuclear cells (Deguchi and Kishimoto, 1991). Subsequently, TGF-β has been detected in the sputum of asthmatics (Adachi *et al.* 1996) and in the BAL fluid of asthmatics both basally and at 24 h following allergen challenge (Redington *et al.* 1997). It has been reported that TGF-β contributes to airway inflammation via macrophages (Prieto *et al.* 2000), neutrophils (Chu *et al.* 2000), and eosinophils (Ohno *et al.* 1996, Vignola *et al.* 1996). The increased levels of TGF-β in the BAL fluid of a murine model of asthma were found to correlate with airway hyperresponsiveness, airway inflammation, collagen deposition, subepithelial fibrosis, and IgE/IgG1 responses (Tanaka *et al.* 2001). The TGF-β secreted from CD4+ T cells has been shown to eliminate allergen-induced eosinophilia (Haneda *et al.* 1999). Also, CD4+ T cells genetically engineered to express TGF-β were shown to reverse allergen-induced hyperreactivity and airway inflammation in a murine model of asthma (Hansen *et al.* 2000).

TGF-β is known to induce IL-11 production, which causes nodular mononuclear infiltrates, airway hyperresponsiveness, airway remodeling, subepithelial fibrosis, and airway obstruction (Zheng *et al.* 2001). In a murine model of asthma, TGF-β was shown to suppress Th2-cell induced airway hyperresponsiveness and airway inflammation (Hansen *et al.* 2000). TGF-β is a fibrokinetic cytokine that induces extra-cellular matrix production, such as collagen. Nomura *et al.* demonstrated in the asthmatic airway that TGF-β could stimulate eosinophils to synthesize collagen (Nomura *et al.* 2002). Collagen deposition in the epithelial basement membrane is a feature of airway remodeling, and accumulation of collagen contributes to basement membrane thickening. It was shown that plasmin-released TGF-β could induce airway smooth muscle cells, which synthesize collagen I in an autocrine manner, contributing to irreversible airway remodeling and fibrosis (Coutts *et al.* 2001).

It has been suggested that the development of airway hyperresponsiveness and airway remodeling in asthmatic airways is regulated by the expression of Smad7, intracellular antagonist of TGF-β, in bronchial epithelial cells. A decreased expression of Smad7 leads to an increased susceptibility of TGFβ on bronchial epithelial cells, causing an increased basement membrane thickness, airway hyperresponsiveness and airway remodeling (Nakao *et al.* 2000, 2002). Increased expression of phosphorylated Smad2, the TGF-β signal transducer, was detected in bronchial biopsy specimens from asthmatics and the increased expression correlated with basement membrane thickness (Sagara *et al.* 2002).

5.6 Smad proteins and TGF-β signaling pathways

5.6.1 Smad proteins

Smad proteins are a family of transcription factors that mediate TGF-β signals.The term Smad originates from the founding members of this family, the *Drosophila* protein MAD (Mothers Against Decapentaplegic) and the *Caenorhabditis elegans* protein SMA (Small body size) (Massagué 2000). In general, Smad proteins are about 500 amino acids in length and consist of two globular domains coupled by a linker region (Shi and Massagué, 2003). Smad transcription factor mediates TGF-β signals into the cell nucleus. The action

of the components that transport SMADs to the specific target genes induces a certain target gene or many potential genes to respond to the SMAD (Massagué 2000).

On the basis of structural and functional characteristics, the Smad family can be divided into three groups consisting of eight distinct Smad proteins: the receptor regulated Smad (R-Smad, Smad1, 2, 3, 5, and 8), the Co-mediator Smad (Co-Smad, Smad4), and the inhibitory Smad (I-Smad, Smad6, 7). R-Smads (Smad1, 2, 3, 5, and 8) can be directly phosphorylated and activated by the type I receptor kinases and form heteromeric complexes with the Co-Smad, Smad4. The activated Smad complexes translocate into the nucleus and, via an interaction with other nuclear cofactors, they regulate the transcription of target genes. Smad1, Smad5 and Smad8 mediate signals downstream of bone morphogenetic proteins (BMP), whereas Smad2 and Smad3 mediate signals triggered by TGF-βs and activins (Leivonen and Kähäri, 2007). The I-Smad (Smad6 and 7) compete with R-Smads for receptor binding or for Co-Smad interaction and they are also involved in targeting the receptors for degradation (Shi and Massagué, 2003). Smad7 is a general inhibitory Smad capable of inhibiting signals triggered by TGF-βs, activins and BMPs, whereas Smad6 inhibits only signals triggered by BMPs (Leivonen and Kähäri, 2007).

Table 3 *The mammalian Smad family members can be divided into three groups according to their function. R-Smad, receptor regulated Smad; Co-Smad, common-mediator Smad; I-Smad, inhibitory Smad; BMP, bone morphogenetic protein.*

R-Smads (Smad1, 2, 3, 5, 8)	Receptor-activated Smads mediating signals triggered by TGF-βs and activins (Smad2, 3) or BMPs (Smad1, 5, 8).
Co-Smad (Smad4)	Forms heteromerix complexes with R-Smads.
I-Smads (Smad6, 7)	Inhibitory function by competing with R-Smads for receptor or Co-Smad interaction or by directing TGF-β receptors for degradation.

5.6.2 Smad-dependent pathways in TGF-β signalling

TGF-β/Smad -signalling is a linear signalling pathway; TGF-β induces signalling responses from the type II to the type I receptor kinase evoking Smad activation causing ligand-induced transcription. TGF-β binds to membrane receptors that have a cytoplasmic serine/threonine kinase domain. Binding of the ligand leads to the assembly of a receptor complex that phosphorylates proteins of the SMAD family. Phosphorylation causes the movement of SMADs into the nucleus where there is the formation of the complexes that directly control gene expression (Massagué 2000). Each ligand of the TGFβ family binds to specific pairs of receptor serine/threonine kinases, which belong to groups type I (TβR-I, ALK5) and type II (TβR-II) receptors. Most mammalian cells express different members of this receptor family, some of which may be shared by different TGF-β ligands (Massagué 2000). The ligand binding causes two type I and two type II receptors to dimerize. Receptor II is known to have one function; to activate receptor I by phosphorylation of the regulatory region in the TGF-β receptor (Wrana *et al.* 1994). Subsequently, the type I receptor phosphorylates SMAD proteins and in this way increases their affinity for Co-Smads. The resulting Smad-complex moves into the nucleus and is able to associate with transcriptional co-activators or co-repressors. In the absence of phosphorylation, Smads are transcriptionally inert (Massagué and Gomis, 2006). The Smads have some affinity for DNA, however, effective binding to particular gene regulatory sites is potentiated by specific DNA-binding cofactors (Massagué 2000). R-Smads that have moved into the nucleus may return to the cytoplasm, though their ubiquitylation- and proteasome-dependent degradation in the nucleus is one way to terminate TGF-β responses (Lo and Massagué, 1999). Activated Smad proteins are recognized by protein phosphatases and ubiquitin ligases that terminate the signaling process. It has been reported that under conditions of TGF-β stimulation, Smads constantly undergo cycles of receptor-mediated phosphorylation and phosphatase-mediated dephosphorylation, moving in and out of the nucleus (Inman *et al.* 2002).

Smad signaling is essential for most, but not all, TGF-β gene responses. TGF-β activates other signalling cascades including the mitogen-activated protein kinase (MAPK) pathway. In addition, a variety of mediators such as ERK, Jun N-terminal kinase (JNK),

p38 MAPK kinase, phosphatidylinositol-3-kinases (PI3Ks), protein phosphatase 2A (PP2A phosphatases) and Rho family members can be activated. Some of these pathways regulate Smad activation, but it is also possible that they can induce responses unrelated to transcription (Derynck and Zhang, 2003).

5.6.3 Smad3 deficiency in mice

Smad3 plays a key role in TGF-β/Smad-mediated transcriptional regulation. Smad3 has been indicated to be a critical effector in TGF-β-mediated cellular responses and the modulation of the activity and the function of the immune system (Datto *et al.* 1999). Phosphorylated Smad3 forms a complex with Smad4, together they are translocated into the cell nucleus to regulate the transcription of target genes (Letterio and Roberts, 1997).
Adult Smad3-deficient mice exhibit impaired mucosal immunity and diminished T cell responsiveness to TGF-β, as well as a lower rate of bone formation and osteopenia through dysregulation of osteoblast differentiation and apoptosis (Yang *et al.* 1999, Borton *et al.* 2001). Smad3-null mice also display an increased rate of wound healing and an impaired local inflammatory response (Ashcroft *et al.* 1999). Mice lacking Smad3 are protected against various cutaneous injuries (Flanders *et al.* 2002, Flanders *et al.* 2003, Saika *et al.* 2004). It has been reported that Smad3-deficiency can increase the incidence of metastatic colorectal tumors (Zhu *et al.* 1998).

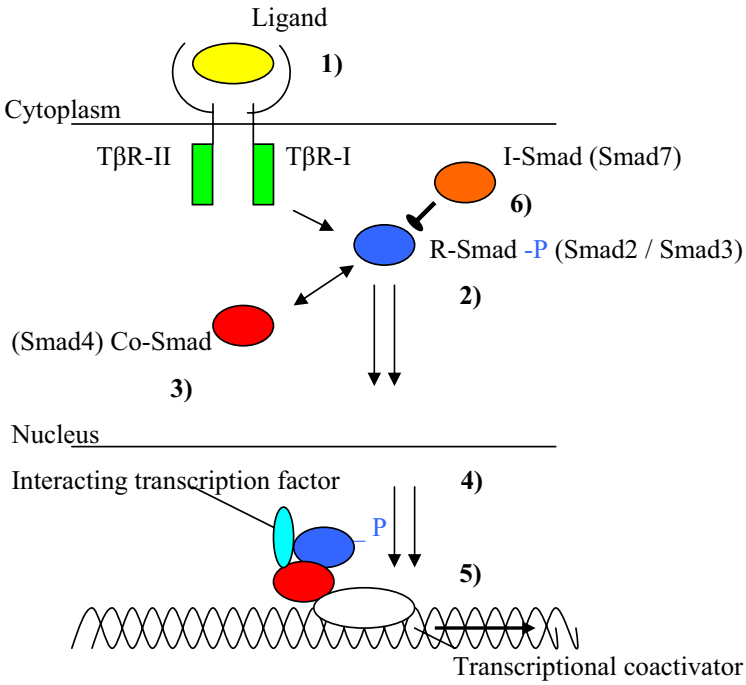

Figure 3 *TGF-β/Smad signalling pathway. 1) Ligand induced receptor activation, 2) R-Smads are phosphorylated, 3) R-Smads and Co-Smads form heteromeric complexes and 4) translocate into the nucleus. 5) Smads control the expression of target genes in a cell type specific manner.6) I-Smads are capable of inhibiting the phosphorylation of R-Smads. I-Smads compete with R-Smads for receptor or Co-Smad interaction. They also direct TGF-β receptors to degradation. R-Smad = receptor-activated Smad, Co-Smad = common-partner Smad, I-Smad = inhibitory Smad, TβR-I = TGF-β type I receptor, TβR-II = TGF-β type II receptor.*

6. AIMS OF THE STUDY

This thesis concentrates on studying the role of a specific transcription factor in TGF-β -signalling pathway in allergic diseases. The role of TGF-β-Smad3 -signalling in the immunoregulation and pathophysiology of allergic disorders is unknown. In this thesis, the effects of Smad3 deficiency have been studied using different animal models in allergic diseases and Smad3 knockout mice.

The specific aims of this study were:

1. To investigate the mechanism in inflammation caused by the defect in TGF-β -Smad3 -signalling in an experimental model of atopic dermatitis (I).

2. To evaluate the role of TGF-β -Smad3 -signalling in the regulation of lung functions in the healthy situation as well as in the disease state using a mouse model of asthma (II).

3. To determine the immunological and histological changes caused by contact allergen and the significance of TGF-β -Smad3 -signalling in a mouse model of contact allergy (III).

4. To investigate the role of TGF-β -Smad3 -signalling and immunological changes after exposure to respiratory allergen in an experimental model of contact allergy (IV).

7. MATERIALS AND METHODS

7.1 Smad3 knock-out mice

Smad3$^{ex8/ex8}$ knock-out mice (Smad3-/-) were provided by Dr. Chuxia Deng (NIH, Bethesda, MD, USA) and were further bred in the facilities of Finnish Institute of Occupational Health. Smad3-/- mice, seven- to 11-weeks of age were used in the experiments. Age and sex matched wild type (WT) offspring were used as littermate controls. All mice were housed in pathogen-free facilities. All animal experiments were approved by Social and Health Services of Finland, Provincial Office of Southern Finland (I - IV).

7.1.1 Breeding and genotyping (I - IV)

Heterozygous Smad3-/- mice were breeded and the resulting progeny were genotyped to identify Smad3-/- and WT mice: A short piece of tail, about 0.5 cm long, was clipped from each mouse to extract DNA. The tail was digested in the presence of protease K (100 µg/ml) in a buffer consisting of 10 mM Tris (pH 8.0), 100 mM NaCl and 1 mM EDTA at 55 °C overnight. After centrifugation for 5 min at 13 000 rpm, the supernatant was collected and boiled at 100 °C for 20 min. The resulting solution (5 µl per 25 µl reaction volume) was used for genotyping by the standard PCR method using mouse primer pairs located in exons 6 and 9 revealing a fragment of 284 bp in Smad3$^{ex8/ex8}$, and 431 bp in WT mice. To detect the wild type (431 bp fragment) the primer pair (5-CCACTTCATTGCCATATGCCCTG-3) and (5-CCCGAACAGTTGGATTCACACA-3) was used and to detect the mutant Smad3 (284 bp fragment) the primer pair (5-CCACTTCATTGCCATATGCCCTG-3) and (5-CCAGACTGCCTTGGGAAAAGC-3) was used. The PCR was performed in a Eppendorf Mastercycler gradient thermal cycler (Perkin-Elmer Corporation, Foster City, CA, USA) using the following cycling parameters: 1) 95 °C, 3 min; 2) 94 °C, 45 seconds; 60 °C, 45 seconds; 72 °C, 1 min; repeat for 32 cycles; 3) 72 °C, 10 min; 4) store at 4 °C. A 284 base pairs (bp) DNA band, formed by direct splicing from exon 6 to exon 9 (Yang *et al.* 1999) and a 431 bp DNA band, were visualized in a 1.5 % agarose gel stained with ethidium bromide.

7.2 Murine models of allergic diseases

7.2.1 Atopic dermatitis (I)

Sensitization

Epicutaneous treatment and sensitization of mice were performed as described previously (Spergel et al, 1998). Inhalation of Isofluran (Abbott Laboratories, Abbott Park, IL) was used to anaesthetize the mice. The skin on the back of the mice was shaved with an electric razor and the shaved area was tape-stripped four times by transparent adhesive tape (Tegaderm, 3M Health Care, St Paul, MN) to remove hair and to introduce a standardized skin injury in stratum corneum to mimic scratching, a symptom of atopic eczema. A sterile gauze patch (1x1 cm^2) moisturized with 100 µl of 0.1% OVA in saline (OVA group) or 100 µl of 0.9% saline (saline group) was placed on the back skin of the mice being attached with Tegaderm adhesive tape.

The patches were kept in place for 1 week and then removed. Two weeks later, an identical patch was applied to the same skin site. The total experiment contained three 1-wk exposures with 2-wk intervals between each exposure. After the last sensitization week, on sacrifice of the mice, blood and skin biopsies were collected for further analysis. The experiment was performed three times.

7.2.2 Allergic contact reactions

Induction of contact hypersensitivity (III, IV)

Epicutaneous treatment and sensitization of mice was performed according to the method described by Lauerma *et al* (Lauerma *et al.* 1997). Mice were lightly anesthetized with inhalation of Isofluran (Abbott Laboratories, Abbott Park, IL) and sensitized on shaved (4 cm^2) and tape-stripped (three times) back skin with 50 µl oxazolone (10 mg/ml) or 100 µl trimellitic anhydride (TMA) (500 mg/ml) in a 4:1 acetone:olive solution on day 0. A control group was treated similarly with only vehicle.

Elicitation (III, IV)

On day 7, the mice were shaved on the back and 50 µl oxazolone (1 mg/ml) or TMA (250 mg/ml) in a 4:1 acetone:olive solution was applied onto the skin. Before allergen application on the back of both ears, the thickness of ears was measured with a micrometer to obtain the baseline level. After the measurement, oxazolone (50 µl, 1 mg/ml) or TMA (50 µl, 250 mg/ml) was applied topically on each ear. In the experiment with oxazolone, the thickness of ear was measured 24 and 48 hours after allergen application. The mice were sacrificed after the last sensitization and ear draining lymph nodes, ears and skin biopsies were collected for further analysis.

Second elicitation (IV)

On day 14, the mice were shaved on the back (no tape stripping) and 50 µl TMA (250 mg/ml) was reapplied on the skin in a 4:1 acetone:olive solution. The thickness of ears was measured with a micrometer before TMA application for baseline level. After the measurement, TMA (50 µl, 250 mg/ml) was applied on the back of both ears topically. The thickness of ear was measured 1, 2, 4, 24 and 48 hours after allergen application. The mice were sacrificed after the last timepoint. Ear draining lymph nodes, ears and skin biopsies were collected for further analysis.

Sample collection and preparation (III, IV)

Skin biopsies from treated skin areas and one ear draining lymph node were used for RNA isolation. The size of the other ear draining lymph node was measured and used for the proliferation test. The ears were weighed and measured with electronic digital micrometer at 0 h, 24 h, and 48 h after oxazolone treatment and at 0 h, 1 h, 2 h, 4 h, 24 h, and 48 h after the TMA treatment.

7.2.3 Asthma (II)

Induction of Allergic Airway Disease

The short-term asthma model was used in this thesis. Mice were sensitized intraperitoneally on day 0 and 10 with ovalbumin in alum or PBS emulsified in alum. On days 20-22, the mice were challenged with aerosolized 1% OVA, for 20 minutes per day. Airway responsiveness was measured 24 hours after the last airway challenge and specimens were collected.

Challenge of eight mice per group was performed in three separate experiments. In addition to the treated mice, the samples from eight naive Smad3-/- mice and eight, age and sex matched, naive WT mice were collected.

7.3 Histology and immunohistochemistry

7.3.1 Skin

Sample collection and skin preparations (I, III, IV)

The collected blood was used for antibody analysis. Skin biopsies from treated skin areas were used for RNA isolation, histology and immunohistochemical examination. For histology, the skin was fixed in 10% formalin, embedded in paraffin wax, sectioned 5 um thick, and stained with H&E and with Periodic Acid Schiff (PAS) stain and examined under light microscopy. For immunohistochemical staining, the skin was embedded into Tissue-Tek OCT compound (Sakura, AA Zoeterwoude, The Netherlands) and quick frozen. The skin samples were cut into 5 um sections and stained immunohistochemically with monoclonal antibodies against CD11c, CD3, CD4 or CD8 (BD, Pharmingen, San Jose, CA) using the ChemMate (DakoCytomation, Glostrup, Denmark) staining kit.

7.3.2 Lung

Cell recovery and histology of lungs (II)

The bronchoalveolar lavage was stained with May-Grünwald-Giemsa (MGG) stain and the cells were counted. The remaining cells were fixed in ethanol (1:2). One part of the left

lung was removed for RNA isolation. The right lung was fixed in 10% formalin, embedded in paraffin wax, sectioned 5 um thick, and stained with H&E and Periodic Acid Schiff (PAS) and examined under light microscopy.

7.4 Molecular biology (I - IV)

RNA isolation and cDNA synthesis

Skin biopsies of the sacrificed mice, obtained at 48 hours after the second allergen exposure, were snap frozen on dry ice and kept at -80°C until homogenized with an Ultra-Turrax T8 (IKA Labortechnik) in TRIzol (Invitrogen Life Technologies). RNA extraction was performed according to TRIzol instructions, followed by DNAseI (RNase-free; Invitrogen Life Technologies) treatment to remove contaminating genomic DNA, and further extraction with phenol-chloroform-isoamylalcohol (25:24:1). MultiScribe reverse transcriptase and random hexameres (Applied Biosystems) were used to reverse transcribe one microgram of extracted RNA into cDNA.

Real-time quantitative PCR

Total RNA from skin or lung was extracted and transcribed into cDNA. Real-time quantitative PCR was performed with an AbiPrism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). The PCR primers and probes were TNF-α, IL-6, IL-1β, IL-4, IFN-γ, IL-13, IL-10, GATA-3, STAT-4, Foxp3, IL-17, TGF-β_1, CXCR3, CCR3, CCR5, CXCL10/IP-10, CCL3/MIP-1α, CXCL5/LIX, CXCL9/MIG, CCL24/eotaxin-2 (Applied Biosystems) and endogeneous 18S rRNA was used as the housekeeping gene and the target gene expression was expressed as relative quantities.

7.5 Immunochemistry (I, II, IV)

ELISA

OVA-specific IgE, IgG_1 and IgG_{2a} serum levels were measured by ELISA method (I, II). Ninety-six-well microtiter plates (Nunc, Rochester, NY) were coated with 100 μg/ml OVA in 0.05 M $NaHCO_3$ (pH 9.6) at 4°C overnight. PBS-Tween 20 (0.05%) was used for

washing and the plates were blocked with 3% BSA in PBS for 2 hours at 20°C and washed again. One hundred microliters of diluted sera (1/10, 1/20, 1/40, 1/80) in 1% BSA-PBS were incubated overnight at 4°C. After washing, 2 µg of biotin-conjugated rat anti-mouse IgE mAb in 1 ml of 1% BSA-PBS was incubated for 2 h at 20°C and washed again. Streptavidin-HRP (BD, Pharmingen, San Jose, CA) 1/4000 in 1% BSA was added and incubated for 30 min at 20°C and washed. After addition of peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), the absorbance was read at 405 nm with ELISA reader (Multiskan MS, Labsystems, Vantaa, Finland). OVA-specific IgG_{2a} was measured by using the same method described. The plates were coated with 2 µg/ml OVA in 0.05 M $NaHCO_3$ (pH 9.6). Serial dilutions of sera for IgG_{2a} (1/60, 1/180, 1/540, 1/1620) were used. Bound IgG_{2a} was detected with biotin-conjugated rat anti-mouse IgG_{2a} mAb (BD, Pharmingen, San Jose, CA).

TMA-specific IgE and IgG_{2a} serum levels were measured by ELISA method (IV). First, TMA-mouse serum albumin (MSA) -conjugate was prepared; TMA in acetone (117 mg/ml) was added (10 µl/10 ml) to the cooled solution of MSA in 0.05 M $NaHCO_3$ (500 µg/ml, pH 9.0). The mixture was allowed to react on ice for 60 min. The conjugates were checked by high-performance liquid chromatography (HPLC) and purified from unreacted components and acetone using Centricon column equilibrated with 50 mM carbonate buffer at pH 9.6. The TMA-MSA conjugate was used for coating microtiter plates (Nunc, Rochester, NY) with 10 µg/ml TMA-MSA conjugate in 0.05 M $NaHCO_3$ (pH 9.6) at 4°C overnight. The plates were washed with PBS-Tween 20 (0.05%) and blocked with 3% BSA in PBS for 2 hours at 20°C and washed again. One hundred microliters of diluted sera in 1% BSA-PBS were incubated at 4°C overnight. After washing, 2 µg of biotin-conjugated rat anti-mouse IgE mAb in 1 ml of 1% BSA-PBS was incubated for 2 h at 20°C and washed again. Streptavidin-HRP (BD, Pharmingen, San Jose, CA) 1/4000 in 1% BSA was added and incubated for 30 min at 20°C and washed. Peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and absorbance read at 405 nm with ELISA reader (Multiskan MS, Labsystems, Vantaa, Finland). TMA-specific IgG_{2a} was measured by using the same method described above. Serial dilutions of sera for IgG_{2a} were used and bound IgG_{2a} was detected with biotin-conjugated rat anti-mouse IgG_{2a} mAb (BD, Pharmingen, San Jose, CA).

Measurement of cytokines by Luminex^{TM} (II)

IL-13, IL-5 and IL-4 cytokines of BAL-samples were measured on a Luminex^{TM} (Bio-Plex 200 System) using the Bio-Rad mouse cytokine kit (Bio-Plex cytokine assay) following the manufacturer's instructions. A minimum of 100 beads per analyte was used for the data collection using Bio-Plex Manager Software (Bio-Rad Laboratories, Inc, Hercules, CA).

7.6 Cell culture (III, IV)

Proliferation test

Ear draining lymph node cells (10^5 cells per well suspended in 200 ul RPMI-1640) were incubated in 37 °C / 5 % CO_2. Lymph node cells were pulsed for 36 hours with 1 μCi [^{3}H] thymidine per well (Amersham Biosciences Europe, Freiburg, Germany). Incorporated radioactivity was measured in a liquid scintillation counter (Trilux 1450 Microbeta, Wallac, Turku, Finland). Results were expressed as mean counts per minute (cpm) of triplicate wells +/- SEM.

8. RESULTS

8.1. Cutaneous responses in the model of atopic dermatitis (I)

Dermal thickness in OVA -sensitized skin sites of Smad3-/- mice was significantly reduced compared to their WT littermates. The epicutaneous OVA sensitization increased significantly the thickness of dermis and epidermis of WT mice compared to saline treated controls. Similarly, the thickness of the epidermis was enhanced in the OVA-sensitized skin of Smad3-/- mice although the difference was not statistically significant.
In the OVA-sensitized skin, the number of mast cells was significantly increased in the Smad3-/- mice compared to WT mice. The mast cell numbers remained at a low level in OVA-sensitized WT mice.
Skin infiltrating eosinophils were significantly increased in OVA-sensitized WT mice compared to saline sensitized controls. The numbers of eosinophils were elevated after OVA sensitization also in Smad3-/- mice compared to their saline treated controls though this was not statistically significant. CD11+ cells were equally elevated in the skin of Smad3-/- mice and their WT littermates after OVA sensitization. In addition, increased numbers of CD3+, CD4+ and CD8+ cells were observed in the skin of Smad3-/- mice and WT mice after epicutaneous OVA sensitization. However, no significant differences in lymphocyte numbers between the Smad3-/- mice and WT mice were detected.

The OVA sensitized skin of Smad3-/- mice expresses drastically lower amounts of IL-6 and IL-1β mRNA compared to their WT littermates. There were no significant differences in the expression of proinflammatory cytokines IL-6, IL-1β and TNF-α mRNA between OVA sensitized and saline sensitized skin in Smad3-/- mice. The mRNA expression of TNF-α, IL-6 and IL-1β was significantly increased in OVA-sensitized skin sites of WT mice compared to saline treated controls.
The expression levels of a major Th2 cytokine IL-4 and Th1 cytokine IFN-γ were significantly higher in the skin of OVA-sensitized mice compared to saline treated controls, but no significant difference between Smad3-/- and WT mice was observed.

8.2. Antibody responses in the model of atopic dermatitis (I)

The OVA-specific IgE response was enhanced significantly in Smad3-/- mice after OVA sensitization compared to their WT littermates. In addition, the levels of OVA-specific IgG2a were induced after OVA sensitization in both mice groups but no significant differences in antibody levels between WT and Smad3-/- mice were detected.

8.3. Cellular infiltration in the lung in the mouse model of asthma (II)

After the last saline or OVA challenge, the mice were killed and the lungs were removed. One part of the lung was stained with hematoxylin and eosin (H&E), and another part with periodic acid-Schiff's reagent (PAS) for PAS -positive cell counting. The numbers of PAS -positive cells were increased in the Smad3-/- animals sensitized with OVA compared to WT mice. Moderate airway inflammation with peribronchiolar and perivascular infiltrates consisting of lymphocytes and eosinophils was seen in the lung sections from Smad3-/- and WT mice sensitized with OVA. The infiltrate of Smad3-/- mice, but not WT mice, consisted of neutrophils. Alveolar macrophage aggregates were detected in Smad3-/- mice, but not in WT mice. In addition, the cell counts of saline sensitized mice were higher in Smad3 -/- mice compared to those of WT mice. The lung histology of naive, unsensitized Smad3-/- mice (3/8), displayed small patch areas with perivascular inflammation and macrophages.

The percentage of lymphocytes was significantly increased in the BAL of Smad3-/- mice compared to WT mice. The amount of eosinophils was increased in the BAL fluid of all OVA-sensitized mice. Neutrophil and macrophage counts in the BAL fluid of Smad3-/- saline treated mice were significantly increased compared to WT saline group.

8.4. Antibody response in the mouse model of asthma (II)

One indication of allergic asthma is the production of allergen specific IgE. Blood was collected after the mice sacrifice and OVA-specific IgE, IgG_1 and IgG_{2a} in the serum were measured by ELISA. OVA-specific IgE levels were increased in Smad3-/- and WT mice sensitized with OVA. Moreover, increased OVA-specific IgG_1 and IgG_{2a} levels in OVA

sensitized Smad3-/- and WT mice were observed, IgG_1 level being slightly higher in WT animals.

8.5. Airway hyperreactivity (II)

Whole-body-plethysmography was used to test mouse airway hyperreactivity (AHR) in response to methacholine after the last OVA or saline sensitization. Smad3-/- OVA-sensitized mice exhibited difficulties in breathing already at lower methacholine concentrations. All three separate experiments displayed a trend in which the PenH pdc value was higher in OVA-sensitized Smad3-/- mice compared to OVA-sensitized WT mice.

8.6 Cytokine and chemokine expression in the lung (II)

IL-13 and IL-5 mRNA levels were significantly increased in OVA-sensitized WT mice compared to OVA-sensitized Smad3-/- mice. The IL-6 level was slightly higher after OVA sensitization in WT mice compared to Smad3-/- mice. The mRNA level of GATA-3 was significantly higher in the lung of naive, unsensitized Smad3-/- mice compared to that of WT mice. Interestingly, naive Smad3-/- expressed also higher levels of TGF-β_1 mRNA compared to naive WT mice. GATA-3 expression was increased also in OVA sensitized WT mice, but not in Smad3-/- mice. The levels of IL-4, IL-10, CXCR3 and CCR4 mRNA were significantly higher in OVA-sensitized animals compared to saline treated animals, but no significant difference between Smad3-/- and WT mice was detected. The same observation was detected in the level of CCR1 mRNA, but the difference was not statistically significant. The differences in mRNA levels of CXCL2 were small between groups. OVA sensitization seemed to increase the mRNA expression of macrophage inflammatory protein 1α (MIP-1α) in the lung. In addition, the protein levels of IL-13, IL-4 and IL-5 in BAL-fluid were examined. IL-13 and IL-5 levels tended to be deceased in OVA-sensitized Smad3-/- mice when compared to WT mice but the differences were not significant. IL-4 level were higher in Smad3-/- mice but again the results were not significant. However, the levels of IL-4 protein were significantly elevated in the BAL of saline treated Smad3-/- mice compared to WT mice. Finally, the basal levels of all Th2 cytokines IL-13, IL-4 and IL-5 were significantly increased in naive Smad3-/- mice as compared to naive WT mice.

8.7 Size of ear draining lymph nodes and cell proliferation in allergic contact reactions (III, IV)

A five fold proliferation of lymph node cells was detected in oxazolone treated mice compared to the control mice. No difference between Smad3-/- and WT mice was detected. The size of ear draining lymph nodes was also significantly increased (approximately four fold) in oxazolone sensitized mice compared to the control groups. However, a significant difference in both the proliferation of ear draining lymph node cells and in the size of ear draining lymph node was detected in TMA treated Smad3-/- mice compared to WT mice. The proliferation of lymph node cells was doubled in TMA treated Smad3-/- mice compared to WT mice and the increased cell count reflected also the doubled size of ear draining lymph node of the Smad3-/- mice. There was a five fold elevation in the proliferation of lymph node cells in TMA treated WT mice compared to the control mice and the size of ear draining lymph node was approximately three fold higher in the TMA treated in comparison with untreated WT mice.

8.8 Ear swelling responses in allergic contact reactions (III, IV)

In the time-course study, the ear thickness was measured 0, 24, and 48 h after the oxazolone challenge. The ear thickness increased with time, but there were no significant differences between Smad3-/- and WT mice. The ear weight was significantly increased in sensitized mice compared to the control animals, but there was statistically no significant difference between WT and Smad3-/- mice. After TMA application, the ear thickness was measured at time points of 0, 1, 2, 4, 24, and 48 h. The ear thickness increased with time, but there was no significant difference between Smad3-/- and WT mice. The ear weight was slightly increased in sensitized mice compared to the control animals, but there were no significant differences between WT and Smad3-/- mice.

8.9 Cell response in allergic contact reactions (III, IV)

A significant increase in the infiltration of neutrophils was detected in oxazolone treated Smad3-/- mice compared to WT mice. The amount of eosinophils, CD4+ cells and the thickness of epidermis was increased upon oxazolone application but no significant difference was detected between Smad3-/- and WT mice. The numbers of skin infiltrating

eosinophils, lymphocytes, and neutrophils were elevated in TMA treated mice. The thickness of epidermis was significantly increased in TMA treated mice and also the thickness of dermis was increased after TMA treatment, this being statistically significant in Smad3-/- mice.

8.10 Chemokine and cytokine skin responses in allergic contact reactions (III, IV)

mRNA expressions of CCL3/MIP-1α and CCL24/eotaxin-2 were dramatically increased in the oxazolone treated skin of Smad3-/- mice compared to WT mice. No evidence of mRNA for CXCL5/LIX was observed in the skin of oxazolone WT mice. The level of CCR3 was significantly increased in oxazolone treated Smad3-/- mice compared to WT mice. The expressions of CCR5, CXCL9/Mig, CXCL10/IP-10 were increased significantly on oxazolone application compared to vehicle control, but no significant differences between Smad3-/- and WT mice were detected.
The levels of proinflammatory cytokines IL-6, IL-1β, and TNF-α and also TGF-β were significantly increased in oxazolone challenged Smad3-/- mice compared to WT mice. Also the expression of a major Th2 cytokine IL-4 was significantly increased in Smad3-/- mice compared to WT mice. In contrast, no significant differences in the mRNA expression of IFN-γ and IL-10 were detected between Smad3-/- and WT mice. T-regulatory cell associated Foxp3 mRNA was significantly increased in oxazolone challenged Smad3-/- mice compared to WT mice.

Also in the skin of TMA sensitized Smad3-/- mice, the expressions of the proinflammatory cytokines IL-6, IL-1β and TNF-α mRNA were increased compared to WT mice. The mRNA for IL-4 was increased in the skin of TMA sensitized Smad3-/- compared to WT littermates, but the difference was not significant. The levels of skin mRNA for IFN-γ and IL-17 were significantly increased in TMA treated Smad3-/- mice compared to WT mice. Also the neutrophil attracting chemokines CCL3/MIP-1α and CXCL5/LIX were significantly increased in TMA treated Smad3-/- mice skin compared to WT mice.

Th1 chemokine response was enhanced in TMA treated mice. CXCL10/IP10 and CXCL9/MIG, Th1 related chemokines, were significantly increased at the mRNA level in

TMA treated mice compared to the control mice. In addition, the mRNA expression of Th1 related chemokine receptor, CCR5, was increased both in WT and Smad3-/- mice, but significantly only in Smad3-/- mice. The Th2 associated chemokine receptor CCR3 was increased upon TMA treatment in WT and Smad3-/- mice, but this was not statistically significant.

8.11 Antibodies after TMA treatment in allergic contact reactions (IV)

TMA-specific IgE levels were increased both in Smad3-/- and WT mice sensitized with TMA, but there were no significant differences in antibody levels between WT and Smad3-/- mice. In addition, the levels of TMA-specific IgG2a were induced after TMA sensitization in both mice groups, however, the levels were significantly decreased in Smad3-/- mice compared to their WT littermates.

9. DISCUSSION

The role of TGF-β-Smad3 -signalling in the immunoregulation and pathophysiology of allergic disorders is still poorly understood. Since TGF-β knock out mice are lethal at early age, using Smad3 knock out mouse in allergy research makes it possible to investigate the significance and role of TGF-β in allergic diseases. Because the role of Smad3 transcription factor has been studied in other diseases, it is possible to utilize this knowledge also in allergy research. The use of murine models either as germline or as tissue specific transgenic mice gives valuable immunological tools to investigate the significance of selected transcription factors or cytokines. Tissue specific transgenic lines have been generated into the Clara cell or CD2 promoter directing tissue- and immune cells specific expression of the gene of interest. DNA microinjection and stem cell transfer, as well as the on/off systems like Cre-lox models help to understand the role of selected genes in various steps of disease pathogenesis. In addition, transgenic models provide information and models for the validation of potential medication, for the development of more efficient and functional compounds as well as preclinical approval of therapy for allergic disease. In this thesis, the various murine models of allergic diseases were used with Smad3-/- mice enabling the investigation of the central TGF-β signalling pathway.

9.1 TGF-β -signaling through Smad3 in the mouse model of atopic dermatitis

Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response (Ashcroft *et al.* 1999). It was anticipated in this thesis, based on the wound repair studies, that a defect in TGF-β -Smad3 -signalling would cause milder skin injury and inflammatory response in the experimental model of AD. Indeed, epicutaneous exposure to OVA in the skin of Smad3-/- mice resulted in significantly reduced dermal thickening and strongly decreased expression of proinflammatory cytokines IL-6 and IL-1β mRNA. However, dermal mast cells and OVA-specific IgE levels were significantly increased in Smad3-/- mice after OVA-sensitization.

Skin thickening, which may be caused by increased collagen deposits, is a characteristic feature of human AD. The pathology of many skin diseases is related to the regulation of the synthesis of extracellular matrix (ECM) molecules. The balance in the synthesis and breakdown of connective tissue is tightly controlled by the release of mediators, including TGF-β, from inflammatory cells or connective tissue cells which can influence collagen and matrix metalloproteinase (MMP) production in both paracrine and autocrine fashions (Eckes *et al.* 2000). Disorders in this system cause wound healing defects or the development of fibrosis. Fibrosis is a normal consequence of chronic inflammation and tissue injury with excessive cell accumulation, skin thickening and deposition of ECM proteins. Many studies show the involvement of Smad3-TGF-β -signalling in the collagen transcription and in the mechanisms of fibrosis (Chen *et al.* 1999; Verrecchia *et al.* 2000). Type I collagen, the major component of ECM is composed of two α1 chains and one α2 chain which are the products of two genes, COL1A1 and COL1A2. After translation, the pro-α1 and pro-α2 polypeptides chains enter into the endoplasmic reticulum where specific proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine. Thereby pro-α chains combine with other chains by hydrogen bonds and form the triple helix procollagen structure. After this procollagens are secreted through the Golgi apparatus in the extracellular space, where the N-terminal and C-terminal propeptides are cleaved by specific proteases. The mature processed collagen molecules aggregates to form larger collagens. Abnormalities in any step of type I collagen production may result in abnormally elevated synthesis of type I collagen which may cause tissue fibrosis (Verrecchia and Mauviel, 2007). Since Smad3 transmits TGF-β signals from the receptor to the procollagen gene in human fibroblasts (Chen *et al.* 1999), it might be that Smad3 regulates collagen production in the murine model of AD used in this thesis. Decreased secretion of collagen in Smad3-/- mice may result in reduced dermal thickness after epicutaneous OVA sensitization.

The proinflammatory cytokines TNF, IL-1 and IL-6 are known to participate in the pathogenesis of AD by orchestrating the interactions between resident and infiltrating cells. In this thesis, it was shown that the expression of skin IL-6 and IL-1β mRNA was strongly decreased in the OVA-sensitized Smad3-/- mice compared to WT mice.

The interaction between IL-6 and TGF-β in disease states has been demonstrated by several studies. An intestinal epithelial cell line study demonstrated a cross-talk between

TGF-β and IL-6, and it suggested that TGF-beta may play a role in the negative regulation of IL-6 signalling in intestinal epithelial cells (Walia *et al.* 2003). Moreover, a study in proximal tubular cells revealed that IL-6 was able to increase TGF-β1 -dependent Smad3-signaling. The study suggested that synergistic effect between IL-6 and TGF-β1 might lead to an enhanced pro-fibrotic response in the kidney (Zhang *et al.* 2005). Furthermore, IL-1β -induced inflammation toward fibrosis was shown to be Smad3 dependent, because it developed only in WT control mice and not in Smad3 null mice (Bonniaud *et al.* 2005).

The role of TGF-β and factors affecting mast cell migration are largely unknown. In this thesis, the increased numbers of mast cells was found in the skin of OVA sensitized Smad3-/- mice suggesting that TGF-β-Smad3 pathway regulates mast cell fate in the allergen sensitized skin. Mast cells, eosinophils, and other cells recruited to the site of inflammation, are potential sources of TGF-β. Smads appear to be involved in TGF-β1-mediated mast cell migration and also TGF-β1-mediated mast cell growth inhibition since TGF-β1 has been shown to induce the migration of the human mast cell line at 40 fM, whereas growth inhibition of mast cells was detected at 400 pM (Olsson *et al.* 2001). Moreover, the signals induced by TGF-β1 that lead to cell migration appear to be different from the signals evoking growth inhibition in mast cells (Olsson *et al.* 2001).). Funaba *et al.* suggested that TGF-β-induced and Smad3-mediated signaling is essential for maximal cell growth in mast cells, and that the activin pathway may be required for it when mast cell context is modulated by Smad3 depletion (Funaba *et al.* 2006).

The elevated levels of IgE and the severity of AD have been shown to correlate (Boguniewicz *et al.* 2006; Flohr *et al.* 2004). In this thesis, the OVA-specific IgE levels, but not OVA-specific IgG2a levels, were significantly upregulated in Smad3-/- mice compared to their WT controls after epicutaneous OVA sensitization. It seems that Smad3-signalling has an inhibitory effect on the production of allergen specific IgE in the model of AD used in the thesis. TGF-β is known to suppress IgE synthesis. Thus, impaired TGF-β-Smad3 signalling may result in the upregulation of OVA-specific IgE antibodies.

9.2 TGF-β -signaling through Smad3 in the mouse models of allergic contact reactions (III, IV)

TGF-β is known to be an important immunomodulatory factor in the progression of DTH reactions and ACD. TGF-β is crucial in regulating the cellular responses involved in contact allergy, such as differentiation, proliferation and migration. T regulatory cells, which produce both TGF-β and IL-10, might play a central role in inducing T cell anergy and active suppression leading to the attenuation of the ACD reaction. However, it is still not clear what are the mechanisms and how TGF-β functions nor is the role of TGF-β / Smad3 -signalling in the immunoregulation of DTH and contact dermatitis totally understood. In this thesis, the role of TGF-β / Smad3 -signalling in a murine model of contact hypersensitivity using Smad3 deficient knockout mice was investigated. Two types of allergens were studied, the contact allergen oxazolone and trimellitic anhydride, the respiratory allergen. TMA inducing stronger Th2, but weaker Th1 responses than OXA, amplified the proinflammatory, Th17 cytokine and regulatory component, TGF-β and Foxp3, respond similarly when Smad3 is lacking. It has been demonstrated that TGF-β secretion inhibits the production of many inflammatory cytokines and chemokines in macrophages including IL-1β, TNF-α, and IL-8 (McDonald *et al.* 1999). In this thesis, significantly increased production of proinflammatory cytokines in mice with TGF-β -signalling deficiency was observed upon sensitization with oxazolone and TMA. IL-6 shares functions with IL-1 and TNF-α, but it also inhibits the synthesis of both of these factors (DiCosmo *et al.* 1994). It is known that adhesion molecules are inducible by stimulation with proinflammatory macrophage-derived cytokines (Bevilacqua, 1993). Furthermore, a certain degree of local trauma can induce or upregulate proinflammatory cytokines, which facilitate, or are required for, the optimal development of skin sensitization. It has been reported that hapten exposure can induce the secretion of proinflammatory cytokines IL-1β, TNF-α and IL-6 in the skin (Bonneville *et al.* 2007, Nakae *et al.* 2001, Enk and Katz, 1992). Dendritic cell-derived IL-1β is critical for the initiation of primary immune responses (Enk *et al.*1993), whereas almost exclusively mast cell derived TNF-α is essential for the recruitment of polymorphonuclear cells and tissue swelling during the effector phase of a CHS response (Biedermann *et al.* 2000). IL-6 is an important activator of T cells, mediator of acute phase reactions, and essential to the CHS response (Hope *et al.* 2000). IL-1β and TNF-α are known to be crucial, as demonstrated

by the impaired CHS in mice lacking these cytokines (Shornick *et al.* 1996, Pasparakis *et al.* 1996). In this thesis, impaired TGF-β signalling due to the lack of Smad3, resulted in a significant increase in the expression of IL-1β, TNF-α and IL-6 in the skin during the CHS response to oxazolone and TMA. The expression of inflammatory cytokine mRNA was significantly increased also in the local draining lymph nodes of allergen sensitized Smad3-/- mice, although at less striking levels than observed in the skin. Since upregulation of proinflammatory cytokine facilitates or is required for the optimal genesis of skin sensitization, the results in this thesis suggest that TGF-beta/Smad3 –signalling is involved in the process of sensitization by regulating the expression of proinflammatory cytokines in the skin.

The general view is that CHS is mediated by IFN-γ producing CD8+ T cells, while IL-2, IL-4 and IL-10 producing CD4+ T cells regulate the response. However, there are also results pointing to an effector role for CD4+ T cell in the CHS response to oxazolone in C57BL6 mice (Xu *et al.* 1996). In addition, the Th2 type cytokine IL-4 might have a proinflammatory role, as shown by the report of impaired CHS responses in mice lacking IL-4 (Traidl *et al.* 1999).

During the immune response to TMA, only low levels of Th1 type cytokines (IFN-γ) are produced, whereas the expression of Th2 cytokines (IL-4) is comparatively high (Warbrick *et al.* 1998). In this thesis, the expressions of both IFN-γ and IL-4 were greatly increased in the skin in Smad3-/- mice compared to WT mice after exposure to topical TMA, suggesting that Smad3 regulates the cytokine expression in both CD4+ and CD8+ T cells, modulating both Th1 and Th2 responses. The level of IL-4, but not IFN-γ mRNA, was greatly increased also in the lymph nodes of Smad3-/- mice after topical TMA exposure. The level of IL-4 mRNA in the skin of Smad3-/- mice was strongly increased after exposure to oxazolone, while the expression of IFN-γ remained at the same level in Smad3-/- mice as in their WT siblings. Thus, IFN-γ production, presumably by CD8+ T cells in this model (Xu *et al.* 1996) is not affected by the lack of Smad3. In contrast, the production of IL-4, mainly by CD4+ T cells, according to previous findings (Xu *et al.* 1996), is strongly affected by the lack of Smad3.

In this thesis the proliferation of ear draining lymph node cells of TMA sensitized Smad3-/- mice increased significantly compared to WT littermates. Also the size of ear draining lymph node was increased in TMA treated Smad3-/- mice compared to WT mice. A recent study claimed that Smad3 was essential for TGF-β blockade of IL-2 production in both CD4+ and CD8+ T cells. There is a report that although TGF-β inhibition of T cell receptor (TCR)-mediated CD4+ T cell proliferation was abrogated in Smad3-/- T cells, its inhibition of CD8+ T cell proliferation remained intact (Bevilacqua, 1993). Thus, in CD8+ T cells, but not in CD4+ T cells, antiproliferative pathways are independent of IL-2 and Smad3. The results in this thesis are in line with the previous data indicating that in WT mice, Smad3 is important for TGF-β inhibition of T cell proliferation. There was a slight tendency toward increased numbers of CD3+ and CD4+ cells in the skin of Smad3-/- mice after oxazolone exposure contributing also to the increased expression of IL-4 mRNA.

It was found that the defect in TGF-β -signalling increased significantly the mRNA expression of both CXCL5/LIX and CCL3/macrophage inflammation protein -1α (MIP-1α) in the skin of oxazolone or TMA treated Smad3-/- mice compared to the WT littermates. The results of the chemokine expression reflected the recruitment of neutrophils in the murine model of contact hypersensitivity. Elevated concentrations of CCL3/MIP-1α and IL-8 have previously been linked to hypersensitivity pneumonitis, an inflammatory lung disease characterized by the presence of CD8+ T cells and neutrophils (Denis, 1995). Murine CXCL5/LIX is a close homolog of human epithelial cell-derived neutrophil attractant-78 (ENA-78, CXCL5) and granulocyte chemotactic protein-2 (GCP-2, CXCL6) (Smith and Herschman, 2004). ENA-78, GCP-2, and IL-8 have been shown to be necessary in the chemotaxis of neutrophils (Rudack *et al.* 2003). IL-8 is one of the most potent chemoattractants for neutrophils. In addition to CCL3/MIP-1α, IL-8 is a powerful stimulating agent for polymorphonuclear neutrophil leukocytes (PMN) (Horuk, 1995). CCL3/MIP-1α is a member of the C-C chemokine supergene family (Schall and Bacon, 1994, Zlotnik *et al.* 1999). Like other members of this family, CCL3/MIP-1α promotes the recruitment of mononuclear phagocytes and lymphocytes (Schall *et al.* 1993, Taub *et al.* 1993). CCL3/MIP-1α has also been reported to be chemotactic for neutrophils both *in vivo* and *in vitro* (Appelberg, 1992). There is a transient appearance of neutrophils at the site of inflammation. After the neutrophils, macrophages participate in the inflammatory

response; they are attracted to the inflammatory site by chemotaxis in the same way as neutrophils. Werner *et al.* showed *in vitro* that TGF-β could inhibit the expression of the inflammatory chemokine CCL3/MIP-1α (Werner *et al.* 2000). The study also suggested that the inhibition of macrophage activation by TGF-β was mediated via Smad3 (Werner *et al.* 2000). This is in line with our result of decreased expression of CCL3/MIP-1α in the mice with deficiensy of TGF-β -signalling. Thus TGF-β / Smad3 -signalling seems to be involved in the regulation of chemotaxis to the site of inflammation.

A recent study suggested a novel role for neutrophils as a source of T cell-recruiting chemokines CXCL10/IP-10 and CXCL9/MIG during the DTH response (Molesworth-Kenyon *et al.* 2005). The CXCR3 activating chemokines CXCL10/IP-10 and CXCL9/MIG have been shown to be expressed in allergic but not in irritant patch test reactions (Flier *et al.* 1999). In this thesis, significantly elevated levels of CXCL9/MIG and CXCL10/IP-10 mRNA were detected in the skin of TMA sensitized mice compared to the control littermates. However, no difference was observed in the expression levels between WT and Smad3-/- mice. The trend reflects the amount of neutrophils detected in this study.

Significantly increased mRNA expression of a major Th2 cytokine IL-4, Th2 related chemokine CCL24/eotaxin-2 and its receptor CCR3, which is expressed predominantly on eosinophils, was observed in the skin of oxazolone challenged Smad3-/- mice compared to WT mice in this thesis. The result may contribute to the increased infiltration of eosinophils seen in the skin of Smad3-/- mice although the difference was not significant. Not only eosinophils but also Th2 lymphocytes and basophils, cells that are important in allergic inflammation, express the CCR3 receptor (Sallusto *et al.* 1997). Therefore CCR3 ligands, such as CCL24, may further amplify the Th2 response in allergic inflammation. In contrast to enhanced Th2 response, the mRNA expression of Th1 related IFN-γ and Th1 linked chemokines CXCL9/MIG and CXCL10/IP-10 were unaltered by the lack of Smad3 in the oxazolone induced contact hypersensitivity response. The present results suggest that TGF-β/Smad3 signaling regulates differently Th1 and Th2 type immune responses. It is possible that Th1 differentiation is more resistant to TGF-β mediated inhibition compared to Th2 differentiation. The exact mechanism of the elevated Th2 type response in the absence of Smad3 however, remains unresolved.

Two chemokines, CCL3 the expression of which is inhibited by TGF-β (Werner *et al.* 2000) and CXCL5, promote the recruitment of neutrophils (Appelberg 1992), which in turn produce the T cell-recruiting chemokines CXCL9 and CXCL10 (Molesworth-Kenyon *et al.* 2005). These chemokines are highly expressed during the CHS response (Flier *et al.* 1999). In this thesis, CCL3 and CXCL5 mRNA levels in the skin of TMA treated Smad3-/- were greatly increased, and the number of neutrophils was slightly, but not statistically significantly increased in the skin of Smad3 deficient mice. The expression levels of CXCL9 and CXCL10 mRNA were equally increased in the skin of TMA exposed Smad3-/- and WT mice. These results suggest that Smad3 is involved in regulating the chemoattraction of neutrophils during the contact sensitivity response to TMA. IL-4 has been reported to have an important regulatory effect on the CHS responses (Asada *et al.* 1997, Weigmann *et al.* 1997). Xu et al demonstrated that IL-4/IL-10 – secreting cells negatively regulate CHS response (Xu *et al.* 1996). It has been demonstrated to block CHS by the inhibition of Langerhans cell migration induced by TNF-α (Takayama *et al.* 1999). Interestingly, in this thesis the expression of skin IL-4 mRNA was increased in the oxazolone and TMA sensitized skin of Smad3-/- mice compared to WT mice. Significantly increased levels of IL-4 and IL-10 mRNA were detected in draining lymph nodes of the ear of oxazolone treated WT mice compared to the WT controls. Instead, in unsensitized Smad3-/- mice, the expression of IL-4 and IL-10 mRNA was increased. Although the expression levels were increased after oxazolone treatment, no significant differences between sensitized and control Smad3-/- ear draining lymph nodes were detected. Kitani *et al* demonstrated that TGF-β -producing regulatory T cells can induce Smad mediated IL-10 secretion and this effect is particularly intense under Th1 conditions (Kitani *et al.* 2003). In addition to IL-4, also Th1 cytokine, IFN-γ, and IL-17 were significantly increased in TMA sensitized Smad3-/- mice compared to WT mice. The skin of oxazolone sensitized Smad3-/- mice expressed increased IL-4 and IL-17 mRNA. Several reports have described that IL-17 can induce the mobilization, recruitment and activation of neutrophils and trigger the production of proinflammatory cytokines and chemokines thereby coordinating local tissue inflammation (Acosta-Rodriquez *et al.* 2007, Kolls and Lindén, 2004). Recent studies have suggested that TGF-β together with IL-6 can mediate the development of IL-17-producing T cells from naive precursors (Veldhoen *et al.* 2006, Bettelli *et al.* 2006, Mangan *et al.* 2006).

TGF-β induces its own gene expression (McCartney-Francis *et al.* 1990). Smad3 plays an important role in the autoregulation of TGF-β expression in fibroblasts and keratinocytes (Ashcroft *et al.* 1999, Piek *et al.* 2001). In the studies of Ashcroft and Piek, Smad3 mutant cells were unable to upregulate their TGF-β expression in response to TGF-β *in vitro*. In the lung, however, a lack of Smad3 was not able to prevent bleomycin-induced upregulation of TGF-β *in vivo* (Zhao *et al*. 2002).
In this thesis, Smad3 deficiency significantly augmented TGF-β expression in the skin.
TGF-β is important for T regulatory cell (Treg) differentiation, maintenance and function (Marie *et al.* 2005) and some studies have implicated TGF-β in the inhibitory mechanisms of Tregs in CHS reactions (Szczepanik *et al*. 2005).

In this thesis, the level of the mRNA expression of TGF-β as well as Foxp3, Treg lineage specific transcriptor factor, was significantly increased in Smad3 deficient skin after topical oxazolone exposure. Treg cells potently suppress T-cell activation. A dysfunction of forkhead box P3 (Foxp3), which controls Treg lineage development, leads to reduced numbers of Treg cells and breaking of immune tolerance, causing aggressive multi-organ autoimmune pathology, often associated with skin inflammation (Dearman and Kimber, 2001, Massague 2000). In mice, depletion of Treg cells has been reported to evoke an enhanced and prolonged Th1-mediated delayed-type hypersensitivity response (Massague, 2000) and adoptive transfer of suppressive Treg cells can reduce the pathology of contact hypersensitivity (Ring *et al.* 2006).
The expression of another important regulatory cytokine, IL-10 (Hawrylowicz and O'Garra, 2005, Taylor *et al.* 2005, Maurer *et al.* 2003), a product of regulatory dendritic cells and Tregs was, however, unaffected by the lack of Smad3. In contrast, topical application of TMA reduced significantly the expression of Foxp3 mRNA in the skin and in the lymph nodes of WT and Smad3 deficient mice.

It was shown in this thesis that TMA-specific IgE levels were increased both in Smad3-/- and WT mice, whereas the level of TMA-specific IgG2a was significantly decreased in Smad3-/- mice compared to their WT littermates. One possible explanation for this phenomenon is the sharp increase in IL-4 production in the Smad3-/- mice, while IFN-γ expression remained at a low level both in the lymph nodes and in the skin. The levels of IgG2a and IgE reflect the Th1 and Th2 responses. The regulation of antibody responses is

of crucial importance in the development of allergy. Although there are many factors that affect antibody production, the predominant signals are provided by Th1 and Th2 cytokines (Dearman and Kimber, 2001). The initiation and maintenance of the IgE response is dependent on IL-4, while IFN-γ antagonizes the production of IgE. The balance between Th1 and Th2 type cytokines also influences the IgG2a response. It has been suggested that the Th1 type response promotes CHS whereas type 2 down-regulates the CHS response (Asada *et al.* 1997). Thus, Smad3 / TGF-β signalling participates in the modulation of the inflammatory hypersensitivity reaction and disease progression through the regulation of the specific antibody response.

Taken together, TGF-β / Smad3 -signaling evokes a wide spectrum of effects in the immune responses of contact allergy. The results in this thesis demonstrate that TGF-β / Smad3 -signalling affects the expression of proinflammatory, Th1 and Th2 cytokines and is involved in the recruitment of cells to the site of inflammation via chemokine secretion. A Smad3 deficiency increases the mRNA expression of CCL3/MIP-1α and CXCL5/LIX in the skin; the increase in these levels correlates with the recruitment of neutrophils in a murine model contact hypersensitivity. The data in this thesis indicates that in WT mice, Smad3 is essential for TGF-β inhibition of T cell proliferation, but in Smad3-/- mice TGF-β is not able to inhibit the proliferation of the T cell subset. TGF-β -signalling modulates Th2 cytokine secretion in the skin and draining lymph nodes of the ear and participates importantly in the regulation of CHS responses. The data demonstrate also that Smad3 can regulate the specific antibody response.

9.3 TGF-β -signaling through Smad3 in the mouse model of asthma

TGF-β is thought to be involved in the development of subepithelial fibrosis, and TGF-β has a major role in the remodelling of the airway wall in bronchial asthma. TGF-β signalling has been shown to be active in asthmatic airways and the activity is associated with the development of airway remodelling in asthma (Sagara *et al.* 2002). A reduced

expression of TGF-β_1 has been associated with an increased eosinophilic inflammation and elevated mucus secretion in response to ovalbumin sensitization (Scherf *et al.* 2005).

In this thesis, it was found that the lack of Smad3-/- signalling led to an exaggerated asthmatic phenotype in an allergic asthma model. The exaggerated asthmatic phenotype seen resembled that reported in the work of Nakao *et al* (Nakao *et al.* 2000) where expression of the inhibitory Smad7 was used to disturb the signalling.

Smad3-/- and WT mice both demonstrated an inflammatory response and, in addition, Smad3-/- mice showed an amplified AHR response to ovalbumin sensitization/challenge in the murine model of asthma used in the thesis. Ovalbumin-specific IgG1 levels were significantly increased in the serum of ovalbumin sensitized Smad3-/- mice compared to the corresponding values in WT mice. The productions of ovalbumin-specific IgE and IgG2a were unaffected by the lack of Smad3, the concentrations were similarly increased in the serum of ovalbumin sensitized Smad3-/- and WT mice. Although the levels of IgG2a were lower in Smad3-/-, the difference was not statistically significant.

Airway hyperreactivity (AHR) in response to the value reflecting methacholine sensitivity, PenH pdc, tended to be higher in OVA-sensitized Smad3-/- mice compared to OVA-sensitized WT mice. Signs of respiratory distress after allergen exposure were observed in Smad3-/- but not in WT mice. However, the differences between groups were not clearly statistically different. This might be explained by the characteristics, as reported earlier (Drazen *et al.* 1996), of the C57BL/6 mouse strain. C57BL/6 mice are more resistant to bronchoconstrictor agents and differences between mice might be difficult to observe with this method.

Th2 cytokines IL-13, IL-5 and IL-4 are closely related to the pathology in asthma. They all play a crucial role in a development of inflammation in this disease. The results in this thesis provide evidence that Smad3 deficiency significantly reduced the expression of IL-13 mRNA in the lung of ovalbumin sensitized allergic mice in the murine asthma model used. The expression of IL-5 was also diminished in the lungs of the ovalbumin sensitized Smad3-/- mice compared to WT mice. Instead, the differences were not detected in the IL-4 mRNA levels between the OVA sensitized Smad3-/- and WT mice. IL-13 is known to have a central role in the effector phases of the Th2 inflammation. IL-13 is a potent inducer of tissue inflammation, airway hyperresponsiveness (AHR), and mucus

hyperproduction (Zhu *et al.* 1999). It has been reported that IL-13 can stimulate airway fibrosis through the action of matrix metalloproteinases on TGF-β and promote epithelial damage and eosinophilia (Cohn *et al.* 2004).
IL-4 is believed to have an important role in the regulation of eosinophilia, AHR and mucus production (Corry 1999). In contrast, blockade of IL-13 signalling was reported to cause a weakening of these features (Grunig *et al.* 1998). In this thesis, Smad3 deficient mice with diminished IL-13 production clearly exhibited increased airway hyperreactivity and substantially increased mucus production. This result is in agreement with the finding of Perkins *et al* (Perkins *et al.* 2006) who demonstrated a role for IL-4 in promoting all the Th2 tissue responses independent of IL-13.

In this thesis, the PAS positive cells were increased in the lung of Smad3-/- mice. The PAS positive cells were detected also in the lung of naive, unsensitized Smad3-/- mice. The results indicate that the TGF-β/Smad3 -signalling is an important to the IL-13 -signalling pathway, and also that TGF-β/Smad3 -signalling mediates mucus production and is able to inhibit PAS production. In contrast to the results in this thesis, the study by Le et al (Le *et al.* 2007) indicated that Smad3 deletion could lead to a decrease in mucin production and eosinophilia whereas no changes with the AHR were detected. However, in the study by Le *et al*, the Smad3 deletion differed from that used in this thesis i.e. exons 2 (Zhu *et al.*1998), instead of exons 8 (Yang *et al.* 1999), were deleted on both alleles. These different Smad3 deletions often give contradictory results. It has been suggested that some part of the mutant transcript (exons 4-9) could be translated in the Smad3 (exon 2) deletion, while in the Smad3 (exon 8) deletion no part of the mutant transcript is translated (Yang *et al.* 1999). Thus, the explanation for the differences between the results in this thesis and the study by Le et al might be that some part of Smad3 had been translated in the latter experiments. The other models utilizing manipulated TGF-β or its receptor report very similar results to the data in this thesis concerning the inflammatory state i.e. eosinophilia, mucin production, and AHR is increased when TGF-β - levels or effect is decreased (Scherf *et al.* 2005, Nakao *et al.* 2000).

The transcription factor, GATA-3, is reported to be the master Th2 regulatory factor and important for Th2-type cytokine production (Zheng and Flavell, 1997, Zhu *et al.* 2004). A

recent study reported that GATA-3 directly could interact with Smad3, and that GATA-3 can mediate the recruitment of Smad3 to GATA binding site. It has been claimed that the two factors cooperate synergistically to regulate transcription from the *Il-5* promoter in a TGF-β-dependent manner (Blokzijl *et al.* 2002). This could explain the result in this thesis of higher IL-5 levels in the lung of OVA sensitized WT mice compared to OVA sensitized Smad3-/- mice. A higher mRNA level of IL-13 in the OVA sensitized WT mice compared to Smad3-/- mice was also detected. Indeed, it has been shown that there are GATA-3-binding sites in the Il-13 promoters, but not in the Il-4 promoter (Lavenu-Bombled *et al*. 2002, Kishikawa *et al.* 2001, Yamashita *et al.* 2002). In the thesis, the mRNA level of GATA-3 was determined in the lung. GATA-3 expression was slightly increased in OVA sensitized WT mice, but not in Smad3-/- mice. It has been reported that a defect in GATA-3 could attenuate allergic inflammation in a murine model of asthma, with decreased eosinophilia, reduced mucus production, and decreased IL-4, IL-5, and IL-13 production (Zhang *et al.* 1999). Similarly, intranasal administration of antisense DNA to GATA-3 reduced airway inflammation, mucus production, and hyperresponsiveness in a murine model of asthma (Finotto *et al.* 2001). It is shown that the deletion of GATA-3 from established Th2 cells abolished IL-5 and IL-13, but not IL-4 production (Zhu *et al.* 2004). Recently, it was demonstrated that GATA-3 can promote Th2 responses through different mechanisms, in collaborating with other transcription factors (Zhu *et al.* 2006). However, there are unknown factors or mechanisms and it is still not known whether Smad3 and GATA-3 cooperate to regulate transcription from the Il-13 promoter in a TGF-β-dependent manner. The findings in this thesis point to a critical dependence on GATA-3 in the way that Smad3 regulates IL-13 expression.

Table 4 *Summary of main results using Smad3-/- mice and different experimental models in allergy research.*

Murine model of AD	**Murine model of asthma**	**CHS studies with contact allergen**	**CHS studies with respiratory allergen**
thickness of dermis reduced in OVA-sensitized skin of Smad3-/- mice	Mucin secretion increased after allergen exposure in Smad3-/- mice	CCL3/MIP-1α and CXCL5/LIX increased in the skin of Smad3-/- mice	Proinflammatory, Th1, Th2 cytokines increased after antigen exposure in Smad3-/- mice
IL-6, IL-1β decreased in OVA-sensitized skin of Smad3-/- mice	AHR increased in Smad3-/- mice after allergen exposure	neutrophil infiltration increased in the skin of Smad3-/- mice	IL-17 increased after antigen exposure in Smad3-/- mice
mast cells increased in OVA-sensitized skin of Smad3-/- mice	IL-13 decreased in the airways of Smad3-/- mice after allergen exposure	CCL24/eotaxin-2, IL-6, IL-1β, TNF-α and IL-4 increased in Smad3-/- mice	
OVA-specific IgE elevated		TGF-β, Foxp3 increased in Smad3-/- mice	
		IFN-γ, CXCL9/MIG, CXCL10/IP-10 unaffected by a lack of Smad3	

10. SUMMARY AND CONCLUSIONS

In this thesis, the role of the TGF-β-Smad -signalling pathway was examined by using Smad3 -deficient mice and evaluated in several murine models of allergic diseases, atopic dermatitis, asthma and contact dermatitis.

In a murine model of atopic dermatitis, the epicutaneous application of ovalbumin (OVA) applied via a patch to tape-stripped skin was used to induce dermatitis in mice (I). The thickness of the dermis was significantly reduced in OVA-sensitized skin of Smad3-/- mice. The defect in the dermal thickness was accompanied with the decrease in the expression of mRNA for proinflammatory cytokines IL-6 and IL-1β in the OVA-sensitized skin. In contrast, the number of mast cells was significantly increased in OVA sensitized skin of Smad3-/- mice and they also exhibited elevated levels of OVA-specific IgE.

The role of TGF-β signalling for the T-helper2 (Th2) cytokine homeostasis in normal lungs and for the regulation of inflammation in a murine model of asthma was studied using mice deficient for Smad3. Allergic airway disease was induced in Smad3 knock-out and wild type mice by sensitizing them by intraperitoneal injections with ovalbumin (OVA), and challenging later with aerosolized ovalbumin (II). Compared to wild type mice, naive (unmanipulated) Smad3-/- mice exhibited significantly increased levels of proinflammatory and Th2 cytokines as well as Th2 associated transcription factor GATA-3 in the lung tissue and bronchoalveolar lavage (BAL). In the asthma model, mucin secretion and airway hyperresponsiveness (AHR) after allergen exposure were significantly increased in the Smad3-/- mice as compared to WT mice. IL-4 levels were equally elevated but IL-13 levels were decreased in the airways of OVA sensitized Smad3-/- mice compared to controls. However, the expressions of IL-4 mRNA and protein were significantly increased in PBS treated Smad3-/- mice. In line with this, the cell counts of saline PBS sensitized mice were higher in Smad3-/- mice and there was an increase in the numbers of eosinophils and neutrophils.

In the contact hypersensitivity studies (CHS) the skin and ears of Smad3 mice were treated with contact allergen, oxazolone (III), or respiratory allergen (IV), trimellitic acid anhydride (TMA).

Topical exposure to oxazolone induced a significantly increased expression of CCL3/MIP-1α and CXCL5/LIX accompanied by increased neutrophil infiltration in the skin of Smad3 deficient mice when compared to their wild type siblings. Also the mRNA expression for CCL24/eotaxin-2, pro-inflammatory cytokines IL-6, IL-1β and TNF-α, as well as Th2 type cytokine IL-4 were significantly increased in Smad3-/- mice compared to wild type mice. Furthermore, topical exposure to oxazolone induced significantly increased expression of TGF-β and Foxp3 mRNA in Smad3-/- mice. However, the expressions of Th1 cytokine IFN-γ, as well as Th1 type chemokines CXCL9/MIG and CXCL10/IP-10 were unaffected by the lack of Smad3, and the ear swelling responses to topically applied oxazolone were similar in Smad3 knockout mice compared to wild type mice.

The defect in TGF-β/Smad3 -signaling promoted the secretion of the proinflammatory, Th1 and Th2 cytokines after topical exposure to TMA. Also the expression of IL-17 mRNA in the skin of TMA sensitized Smad3-/- mice was increased indicating that Smad3 may be involved in the progress of tissue inflammation via IL-17. In contrast to the situation with the contact allergen OXA, the respiratory allergen TMA did not induce Foxp3 expression. The shifted Th1/Th2 balance due to Smad3 deficiency during the CHS response to TMA, also caused modulated antibody class switching.

Furthermore, the mRNA expressions of chemokines contributing to the leukocyte recruitment, CCL3/MIP-1α and CXCL5/LIX, were upregulated in Smad3-/- mice. The data in the thesis is in line with the previous data suggesting that in wild type mice, Smad3 is essential for TGF-β inhibition of T cell proliferation, but in Smad3-/- mice TGF-β is not able to inhibit the proliferation of the T cell subset. This data suggests also that Smad3 regulates the specific antibody response.

In conclusion, Smad3-pathway regulates allergen induced skin inflammation and systemic IgE antibody production in a murine model of AD. TGF-β / Smad3 -signalling contributed to inflammatory hypersensitivity reactions and disease progression via modulation of chemokine and cytokine expression, regulation of specific antibody response and

inflammatory cell recruitment in a murine model of CHS. The defect in Smad3 -signalling decreased the expressions of IL-13 and IL-5 mRNA in the lung, modulated allergen induced specific IgG1 response, and affected mucus production in the lung in a murine model of asthma.

In summary, TGF-β can modulate inflammatory responses - at least partly through the Smad3 pathway - but also through other compensatory, non-Smad-dependent pathways. The Smad3 signalling pathway might be a potential target in the therapy of allergic skin diseases. Unravelling the mechanisms of Smad3 may open new possibilities for treating and preventing allergic responses, which may lead to severe illness and loss of work ability.

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